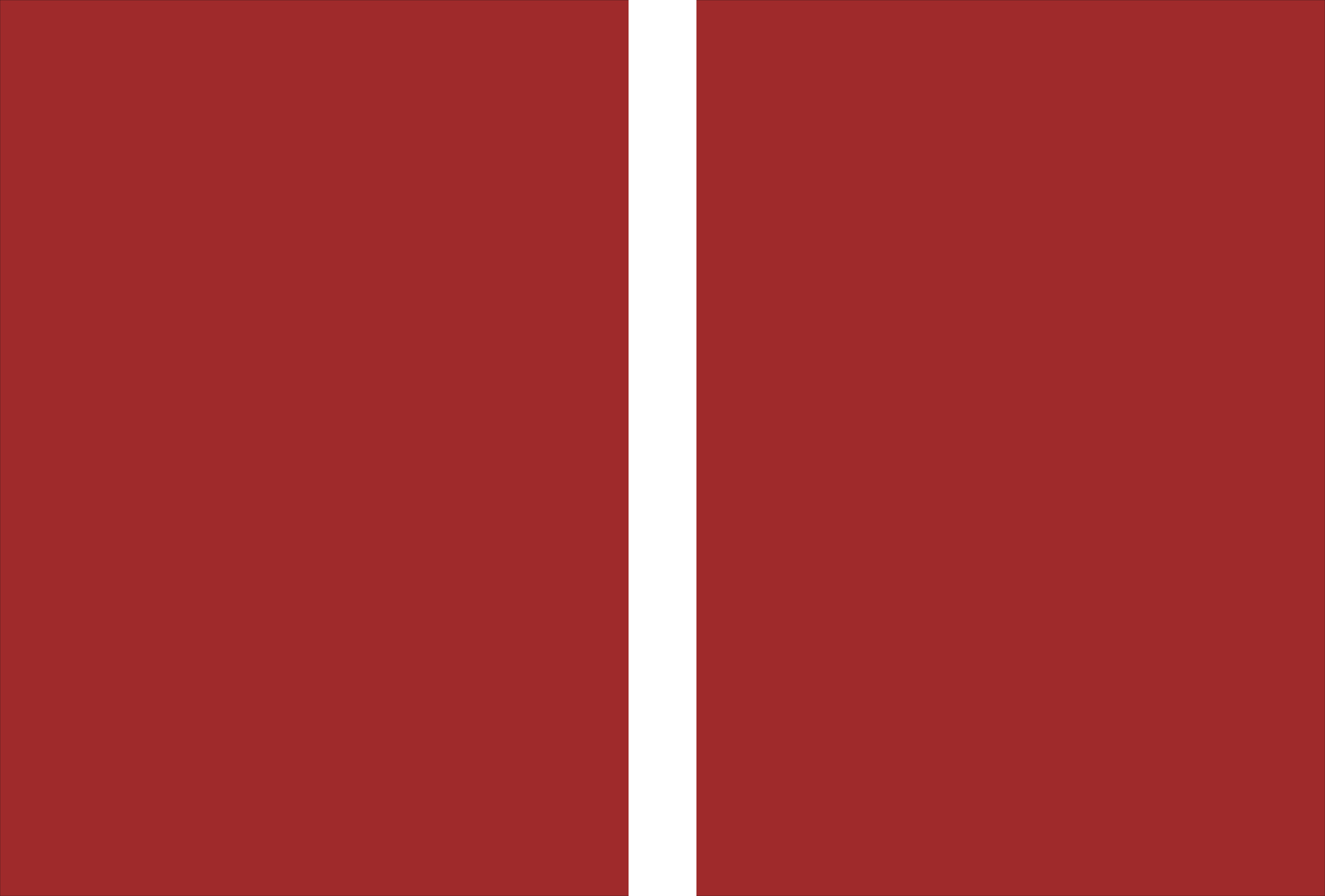


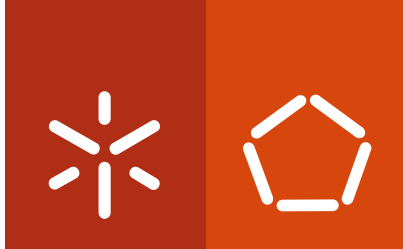


Universidade do Minho
Escola de Engenharia

Nuno André Martins Silva

3D Functionalized Multi-Supportive Structures (FMSS) – A Novel Route for Spinal Cord Injury Regeneration





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Programa Doutoral em Engenharia de
Tecidos, Medicina Regenerativa e Células
Estaminais

Trabalho realizado sob a orientação do
Professor Doutor Rui L. Reis
e co-orientação do
Doutor António J. Salgado

Julho de 2012

É AUTORIZADA A REPRODUÇÃO PARCIAL
DESTA TESE APENAS PARA EFEITOS DE
INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO
ESCRITA DO INTERESSADO, QUE A TAL SE
COMPROMETE

Universidade do Minho ____/____/____

Nuno André Martins Silva

TO MY PARENTS

"Don't give up. Don't lose hope. Don't sell out. "

Christopher Reeve

(1952-2004)

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3D Functionalized Multi-Supportive Structures (FMSS) – A Novel Route for Spinal Cord Injury Regeneration

ABSTRACT

A deregulation or injury in the nervous system usually leads to devastating consequences. In the particular case of spinal cord injury (SCI), patients have to deal with several neurological deficits and disabilities. Therefore, it is urgent to develop therapeutic strategies that can specifically target this problem. Different possible solutions have been experimented to solve these conditions, such as cell and molecular therapies, although the outcomes are still not satisfactory, imposing a need for alternative approaches. Tissue engineering has been proposed as a new method to address these problems. The tissue engineering strategy usually implies the use of a 3D structure that is able to support the cell growth and differentiation in an adequate environment towards the development of a functional tissue engineered system. With the concepts of tissue engineering in mind, the main goal of the work described in this thesis was to develop a structure that can be easily, efficiently, and successfully applied in the treatment of spinal cord lesions.

In the work presented in this thesis, a natural blend of starch/poly- ϵ -caprolactone (SPCL) was processed by rapid prototyping in order to create a tubular porous scaffold. Then, these SPCL scaffolds were filled with the gellan gum hydrogel with the purpose of creating a novel hybrid structure. In this biphasic scaffold the SPCL phase aims to mimick the vertebra bone functions, while the gellan gum hydrogel is designed to be a cell encapsulation system to support axonal regeneration in the injured spinal cord. The developed scaffolds presented tunable outer layer thickness, mechanical modulus, fiber/filament orientation, and pore geometry. Moreover, the scaffolds disclosed a non-

cytotoxic behavior and allowed the *in vitro* culturing of oligodendrocytes, olfactory ensheathing cells (OECs) and Schwann cells for periods up to three weeks. In order to overcome some of the drawbacks associated with the use of gellan gum, namely the absence of biological signals for cellular adhesion and proliferation, this hydrogel was chemically conjugated with the peptide sequence GRGDS, via Diels-Alder click chemistry. It was observed that the novel GRGDS-gellan gum had a profound effect on neural stem cell morphology and proliferation. These results demonstrated the importance of GRGDS for cell-gellan gum interaction. Subsequently, the *in vivo* experiments revealed that hybrid structures are biocompatible. However, more importantly, *in vivo* tests showed that SPCL are suitable structures to promote spine stabilization and that the stabilization of SCI rats was associated with functional motor recovery. Finally, in the scope of this thesis, it was also assessed the effects of OECs secretome on the growth of adult stem cells derived from adipose, bone marrow, umbilical cord and nervous tissue. Results revealed that OECs secretome increase the metabolic activity and/or proliferation of these adult stem cells.

As a concluding remark, it can be stated that the work described in this thesis brings new knowledge to the cell biology, biomaterial and spinal cord injury fields. The results obtained in the *in vivo* experiments indicate that researchers currently testing treatments for SCI repair might have to take into account the use of spine stabilization in combination with their approaches. From the biomaterial point of view, the simplicity and broad applicability of the Diels-Alder click chemistry (used to functionalize the gellan gum) can be easily extended to other molecules to further improve this material. Finally, important understanding was herein created about biological/molecular interactions between OECs and adult stem cells from several origins.

Estruturas de Suporte Multifuncionais: Uma Nova Terapia para Regeneração de Lesões da Medula Espinal

RESUMO

Uma lesão do sistema nervoso acarreta normalmente consequências devastadoras. No caso particular de lesões da medula espinal (LME), os pacientes têm que lidar com múltiplas insuficiências biológicas. Por este motivo, é urgente desenvolver terapias que possam resolver eficazmente este problema. Diferentes estratégias têm sido propostas para tratar esta condição, tais como, terapias baseadas em transplantação celular e/ou terapias moleculares. No entanto, os resultados não têm sido satisfatórios, reforçando a ideia que são necessárias abordagens alternativas. A engenharia de tecidos tem sido proposta como um novo método para enfrentar estes problemas. Esta estratégia implica o uso de uma estrutura 3D que é capaz de suportar o crescimento e a diferenciação celular de forma a criar um ambiente adequado para o desenvolvimento de um tecido funcional. Tendo por base os conceitos da engenharia de tecidos, o principal objectivo do trabalho descrito nesta tese foi o desenvolvimento de um sistema que possa ser aplicado facilmente e eficazmente no tratamento de LME.

Neste trabalho, o polímero natural de poli-caprolactona de amido (SPCL) foi processado por prototipagem rápida de forma a criar estruturas tubulares porosas. Seguidamente, o interior das estruturas de SPCL foram preenchidos com o hidrogel gellan gum de forma a criar uma estrutura híbrida. Neste sistema, o SPCL tem como objectivo mimetizar as funções do osso vertebral. Por sua vez, o hidrogel tem como objectivo servir como um sistema de encapsulamento celular de forma a suportar a regeneração nervosa. As estruturas desenvolvidas possuem espessura, orientação e geometria do poro e características mecânicas configuráveis. Adicionalmente, as

estruturas apresentaram um comportamento não tóxico e permitiram o crescimento *in vitro* de oligodendrócitos, células do bulbo olfativo (OECs) e células de Schwann. De forma a ultrapassar algumas das desvantagens do gellan gum, nomeadamente o facto de este material não possuir na sua estrutura sinais biológicos, este material foi modificado com o péptido GRGDS. Foi observado que o GRGDS-gellan gum tem um forte efeito na morfologia e proliferação de células estaminais neuronais. Estes resultados demonstraram a importância do péptido GRGDS na interacção célula-gellan gum. Seguidamente, as experiências *in vivo* revelaram que estas estruturas são biocompatíveis, mas, mais importante, os testes em animais demonstraram que as estruturas de SPCL permitem a eficaz estabilização da coluna vertebral e que esta estabilização promove a recuperação da função motora de ratos com LME. Finalmente, no âmbito do trabalho descrito nesta tese, foi também estudado o efeito que os factores secretados pelas OECs exercem sobre a proliferação e/ou actividade metabólica de células estaminais adultas derivadas do tecido adiposo, da medula óssea, do cordão umbilical e do tecido nervoso. Os resultados revelaram que as OECs produzem factores que promovem o aumento da actividade metabólica e/ou proliferação dessas células estaminais adultas.

O trabalho descrito nesta tese originou novo conhecimento para as áreas de biologia, de biomateriais e de regeneração de LME. As experiências *in vivo* revelaram que os investigadores que estejam actualmente a testar terapias para LME devem ter em conta a estabilização vertebral. Do ponto de vista dos biomateriais, a modificação do gellan gum é simples e de ampla aplicabilidade. Neste sentido, o gellan gum pode agora ser facilmente modificado com diferentes moléculas consoante a aplicação desejada. Finalmente, o entendimento sobre o efeito dos factores produzidos pelas OECs em células estaminais, poderá revelar-se importante para futuras terapias celulares.

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LIST OF ABBREVIATIONS

#

2D - two dimensional
3D - three dimensional
5-HT - 5-hydroxytryptamine

A

A – Angstrom
AANS/CNS - American Association of
Neurological Surgeons/Congress of
Neurological Surgeons
A/B - antibiotic
ANOVA - analysis of variance
ASC - adipose stem cells
ATP - adenosine-5'-triphosphate

B

BBB – blood brain barrier or Basso,
Beattie and Bresnahan test
BDA - biotinylated dextran amine
BDNF - brain-derived neurotrophic
factor
bFGF - basic fibroblast growth factor
BMSCs - bone marrow stromal cells
BrdU - 5-bromo-2'-deoxyuridine
BSA - bovine serum albumin

C

CA - cellulose acetate
CaCl₂ - calcium chloride
Ca²⁺ - calcium
CAD - computer assisted design
cAMP - cyclic adenosine
monophosphate
CD11b - cluster of differentiation 11B
ChABC - chondroitinase ABC
cm – centimeter
CNS – central nervous system
CO₂ - carbon dioxide

COOH - carboxylic group
CSPG - chondroitin sulphate
proteoglycan
CST - corticospinal tract
CTB - cholera toxin B subunit

D

D₂O - deuterated water
Da – Dalton
DAPI - 4',6-diamidino-2-phenylindole
DIPEA - Diisopropylethylamine
DMEM - Dulbecco's modified Eagle's
medium
DMA - dynamic mechanical analysis
DMF - Dimethylformamide
DMTMM - 4-(4,6-Dimethoxy-1,3,5-
triazin-2-yl)-4-methylmorpholinium
chloride
DNA - deoxyribonucleic acid
dsDNA - double-stranded
deoxyribonucleic acid
DSPG - dermatan sulphate proteoglycan

E

ECACC - European Collection of Cell
Cultures
ECM - extracellular matrix
EGF - Epidermal growth factor
ELISA - Enzyme-Linked
Immunoabsorbent Assay
EPO - erythropoietin
ESCs - embryonic stem cells
EVOH - ethyl vinyl alcohol

F

FBS - fetal bovine serum
FDA - Food and Drug Administration

FIM - Functional Independence Measure
fMRI - functional magnetic resonance imaging
FMSS – Functionalized Multi-Supportive Structure
FORE-SCI - Facilities of research excellence in spinal cord injury

G

GABA - gamma - aminobutyric acids
GAG – glycosaminoglycans
GAP43 - growth associated protein 43
G-CSF - granulocyte-colony stimulating factor
GDNF - glial-derived neurotrophic factor
GFAP - Glial fibrillary acidic protein
GG – Gellan Gum
GG-GRGDS – Gellan Gum modified with a glycine-arginine-glycine-aspartic acid-serine sequence
g/L - gram per liter
GM-1 – monosialotetrahexosyl ganglioside
GRGDS – Glycine-Arginine-Glycine-Aspartic acid-Serine
GRPs - glial-restricted precursors

H

H&E - hematoxylin-eosin
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC - high-performance liquid chromatography
HSPG - heparan sulphate proteoglycan
HUCPVCs - Human umbilical cord perivascular cells
Hz - hertz

I

ICC – immunocytochemistry

ICCP - International Campaign for Cures of Paralysis
IGF-I - insulin-like growth factor type I
IL10 - interleukin-10
IN1 - nogo receptor
iPSCs - induced pluripotent stem cells

K

K⁺ - potassium
kDa - kilodalton
KSPG - keratan sulphate proteoglycan
kV – kilovolt

L

L929 - rat lung fibroblasts cell line

M

M - molar
MAG - myelin-associate glycoprotein
MAP-2 - Microtubule-associated protein 2
MEM - minimum essential medium
MES - 2-(N-morpholino)ethanesulfonic acid
min - minute
mg - miligram
Mg²⁺ - magnesium
ml - mililiter
mM - milimolar
mm - milimeter
MO3-13 - human oligodendrocyte cell line
MPa - megapascal
MP – methylprednisolone
MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

N

n - total number of data points
Na⁺ - sodium
NaCl - sodium chloride

NASCIS - National Acute Spinal Cord Injury Study
 N-CAM - Neural Cell Adhesion Molecule
 NEP 1-40 - NgR antagonist
 NF – neurofilament
 NIH - National Institutes of Health
 NGF - nerve growth factor
 nm - nanometer
 NMR - Nuclear magnetic resonance
 NMDA - N-Methyl-D-aspartate
 NRPs - neuronal-restricted precursors
 NSCs - neural stem cells
 NSAIDs - non-steroidal anti-inflammatory drugs
 NT – Neurotrophin

O

O4 - Oligodendrocyte Marker 4
 OD - optical density
 OECs - olfactory ensheathing cells
 OF - Open Field
 OPCs - oligodendrocytes progenitor cells

P

p - probability value
 Pa – pascal
 PA - peptide amphiphile
 PBS - phosphate buffered saline
 PCL - polycaprolactone
 PDGF-AA - platelet derived growth factor AA
 PEG - polyethylene glycol
 PEG-PLA - polyethylene glycol - polylactic acid
 PFA - paraformaldehyde
 PGA - polyglycolic acid
 PHA-L – leucoagglutinin
 PHEMA - poly-(2-hydroxyethyl)-methacrylate

PHEMA-MMA - poly-(2-hydroxyethyl)-methacrylamine-co-methyl metacrilate
 PHPMA - poly-(2-hydroxypropyl)-methacrylamine
 PITC - phenylisothiocyanate
 PLA - Polylactic acid
 PLGA - poly(lactic-co-glycolic)
 PNS – peripheral nervous system
 PTC - phenylthiocarbamyl

R

RGD - arginine-glycine-aspartic acid
 RhoA - Ras homolog gene family, member A
 ROS - reactive oxygen species
 rpm - revolutions per minute

S

s - second
 SCI – Spinal Cord Injury
 SCs - Schwann cells
 SD – Standard deviation
 SEM - scanning electron microscopy or standard error of the mean
 SPARC - secreted protein, acidic and rich in cysteine
 SPCL - starch-polycaprolactone
 SPS - solid-phase synthesis

T

TE - Tissue engineering
 TFA - trifluoroacetic acid
 TGF- α transforming growth factor alpha
 TNBF - trinitrobenzene sulfonate
 TRG - thyrotropin-releasing hormone

U

μ A - microampere
 μ -CT - Micro-computed tomography
 μ m – micrometer

V

VEGF - vascular endothelial growth factor

v/v - volume per volume

W

WGA-HRP - horseradish peroxidase conjugated to wheat germ agglutinin

WJ - Wharton's jelly

Wt - weight

wt/v - weight per volume

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CHAPTER I

From Basics to Clinical: A Comprehensive Review on Spinal Cord Injury

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SHORT *CURRICULUM VITAE*

Nuno Silva was born in Vila Nova de Famalicão, Portugal, in 1985. He is currently a PhD student at the 3B's Research Group (Biomaterials, Biodegradables and Biomimetics) in collaboration with the Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Portugal. His work was performed under the supervision of Prof. Rui L. Reis, the Director of the 3B's Research Group, and Dr. António Salgado, junior PI at ICVS.

Nuno obtained his degree in Applied Biology from the School of Sciences of the University of Minho, graduating with the final score of 15 (0-20 scale). To conclude his degree, he developed his final project as research trainee at the 3B's Research Group University of Minho, Portugal, under the supervision of Prof. Rui Reis and co-supervision of António Salgado and Rui Amandi, where he received a traineeship mark of 19 (0-20 scale).

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Portugal, 2010; Travel Award for attendance at XII International Meeting of the Portuguese Society for Neurosciences, Lisbon, Portugal, 2011; Travel Award from IBRO/FENS for attendance at 8th FENS Forum of Neuroscience, Barcelona, Spain, 2012. He has been an invited reviewer for Current Stem Cell Research & Therapy, Tissue Engineering Part A, Current Biotechnology and Journal of Tissue Engineering and Regenerative Medicine.

He is also a member of the Portuguese Society of Stem Cells and Cellular Therapies since 2008, the TERMIS-EU since 2008, the Portuguese Society for Neuroscience since 2009 and the American Society for Neuroscience since 2010.

As a result of his research efforts he is author or co-author of 11 research papers (6 published and 5 submitted), 1 book chapter and has done more than 20 communications (oral/poster) in international meetings.

LIST OF PUBLICATIONS

The research work performed during this PhD resulted in the following publications:

Peer-Reviewed Journals

Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Pedro AJ, Leite-Almeida H, Cerqueira R, Almeida A, Mastronardi F, Mano JF, Neves NM, Sousa N and Reis RL, “Development and Characterization of a Novel Hybrid Tissue Engineering Based Scaffold for Spinal Cord Injury Repair”, *Tissue Engineering Part A*, 2010, Jan;16(1):45-54

Silva NA, Sousa RA, Oliveira AO, Sousa N, Salgado AJ, Reis RL, “Interactions Between Schwann and Olfactory Ensheathing Cells with a Starch/Polycaprolactone Scaffold aimed at Spinal Cord Injury Repair”, *J Biomed Mater Res Part A* 2012;100A:470–476.

Silva NA, Sousa RA, Fraga, JS, Fontes M, Leite-Almeida H, Cerqueira R, Almeida A, Sousa N and Reis RL, Salgado AJ, “Benefits of Spine Stabilization with Biodegradable Scaffolds in Spinal Cord Injured Rats”, *Tissue Engineering Part C*, 2012, Epub ahead of print.

Silva NA, Cooke MJ, Tam RY, Sousa N, Salgado AJ, Reis RL, Shoichet MS, “The Effects of Peptide Modified Gellan Gum and Olfactory Ensheathing Glia Cells on Neural Stem/Progenitor Cell Fate”, *Biomaterials* 33 (2012), pp. 6345-6354.

Silva NA, Gimble JM, Sousa N, Reis RL, Salgado AJ, “Combining Adult Stem Cells and Olfactory Ensheathing Cells: The Secretome Effect”, Submitted.

Silva NA, Sousa N, Salgado AJ and Reis RL, “From Basics to Clinical: A Comprehensive Review on Spinal Cord Injury”, Submitted

Invited Lectures

Silva NA, Sousa N, Reis RL, Salgado AJ, “Stem Cells and *In Vivo* Animal Models: Spinal Cord Injury”, 2nd Edition of Stem Cells Get Practical: Approaches on Stem Cells Isolation, Characterization and Differentiation, ECS International Postgraduate Programme, Braga, Portugal, March 2012.

Silva NA, Sousa N, Salgado AJ, Reis RL, “Tissue Engineering and Spinal Cord Injury: The Multidisciplinary Approach”, III Conference on Science and Medicine – Regenerative Medicine, Braga, Portugal, April 2012.

International Conferences

Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Mastronardi F, Pedro AJ, Neves NM, Mano JF, Sousa N and Reis RL, "Development of Novel Starch Based 3D Tubular Scaffolds for Spinal Cord Injury Repair: Scaffold Design, Mechanical Properties and Biological Evaluation", 4th Marie Curie Cutting Edge InVENTS Conference on Biocompatibility evaluation and biological behaviour of polymeric biomaterials, Alvor, Portugal, October 2007 (*poster presentation*).

Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Neves NM, Mano JF, Sousa N and Reis RL, Development of a Novel Hybrid Tissue Engineering Based Therapy For Spinal Cord Injury Repair, Third International Meeting of the Portuguese Society for Stem Cells and Cell Therapies, Faro, Portugal, April 2008 (*oral presentation*).

Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Neves NM, Mano JF, Sousa N and Reis RL, Processing and Biological Characterization of Starch/Gellan Gum Hybrid Structures for Spinal Cord Injury Repair, 5th Marie Curie Conference on "Synthesis and Applications of Self Assembling Materials at nano-scale", Funchal, Portugal, April 2008 (*poster presentation*).

Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Mano JF, Neves NM, Sousa N and Reis RL, Starch/Gellan Gum Hybrid 3D Guidance Systems for Spinal Cord Injury Regeneration: Scaffolds Processing, Characterization and Biological Evaluation, TERMIS-EU 2008, Porto, Portugal, June 2008 (*oral presentation*).

Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Fraga JS, Cerqueira R, Leite-Almeida H, Almeida A, Sousa N and Reis RL, From the development to the physical and biological assessment of a novel biodegradable 3D structure for Spinal Cord Injury Repair, 11th Meeting of the Portuguese Society for Neurosciences, Braga, Portugal, June 2009 (*poster presentation*).

Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Fraga JS, Cerqueira R, Leite-Almeida H, Almeida A, Neves NM, Mano JF, Sousa N and Reis RL, Development and Biological Evaluation of a Novel Hybrid 3D Tubular Scaffold for Neural/Bone Regeneration in Spinal Cord Injury, 2nd TERMIS World Congress, Seoul, Korea (south), August 2009 (*poster presentation*).

Silva NA, Sousa RA, Oliveira JT, Fraga JS, Leite-Almeida H, Cerqueira R, Almeida A, Mano JF, Neves NM, Sousa N, Salgado AJ and Reis RL, Novel Starch/Gellan Gum Tubular Scaffolds for Spinal Cord Injury: From the Development to the Physical and Biological Assessment, 5th European Symposium on Biopolymers “Design, Production & Applications of Biopolymers in Tissue Engineering, Regenerative Medicine, Drug Delivery, Biotechnology and Nanomedicine”, Funchal, Portugal, November 2009 (*oral presentation*)

Silva NA, Sousa RA, Oliveira JT, Cerqueira R, Leite-Almeida H, Almeida A, Sousa N, Salgado AJ and Reis RL, Gellan Gum Hydrogel and SPCL Tubular Scaffolds: In Vivo Evaluation on Spinal Cord Injury Repair, 5th Annual International Meeting of the Portuguese Society for Stem Cells and Cellular Therapies, Guimaraes, Portugal, May 2010 (*poster presentation*).

Silva NA, Sousa RA, Oliveira JT, Cerqueira R, Leite-Almeida H, Almeida A, Reis RL, Salgado AJ and Sousa N, In Vivo Assessment of the Regenerative Potential of Two Biodegradable Polymers for Spinal Cord Injury Repair, TERMIS-EU 2010, Galway, Ireland, June 2010 (*oral presentation*).

Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Mano JF, Neves NM, Sousa N and Reis RL, Development, Characterization and In Vitro Biological Assessment of a Novel Hybrid 3D Scaffold for Spinal Cord Injury Repair, TERMIS-EU 2010 , Galway, Ireland, June 2010 (*oral presentation*).

Silva NA, Sousa RA, Oliveira JT, Fraga JS, Cerqueira R, Leite-Almeida H, Almeida A, Sousa N, Salgado AJ and Reis RL, Functionalized Multi Supportive Structures Aimed For Spinal Cord Injury Repair, 2nd Meeting of the Institute for Biotechnology and Bioengineering, Braga, Portugal, October 2010 (*poster presentation*).

Silva NA, Sousa RA, Oliveira JT, Fraga JS, Cerqueira R, Leite-Almeida H, Almeida A, Sousa N, Salgado AJ and Reis RL, A Novel 3D Scaffold for Neuronal Regeneration in Spinal Cord Injury: From Development to In Vitro and In Vivo Characterization, Neuroscience 2010, San Diego, United States of America, November 2010 (*poster presentation*).

Silva NA, Sousa RA, Oliveira JT, Fraga JS, Cerqueira R, Leite-Almeida H, Almeida A, Salgado AJ, Sousa N and Reis RL, Effects of Spine Stabilization on the Motor Improvements of Spinal Cord Injured Rats after the Implantation of a Novel 3D Natural based Biodegradable Scaffold, TERMIS-NA, Florida, United States of America, December 2010 (*poster presentation*).

Silva NA, Sousa RA, Oliveira JT, Fraga JS, Fontes M, Cerqueira R, Leite-Almeida H, Almeida A, Sousa N, Reis RL and Salgado AJ, Motor and Sensory Improvements after Spine Stabilization of Spinal Cord Injured Rats with a Novel 3D Natural based Biodegradable Scaffold, XII Meeting of the Portuguese Society for Neurosciences, Lisbon, Portugal, May 2011 (*poster presentation*).

Silva NA, Sousa RA, Shoichet MS, Sousa N, Salgado AJ, Reis RL, Implantation of a Novel Hybrid 3D Scaffold Improves Motor Behavior of Spinal Cord Injured Rats, 8th FENS Forum of Neuroscience, Barcelona, Spain, July 2012 (*poster presentation*).

The collaborative work within the 3B's and ICVS generated the following publications:

Peer-Reviewed Journals

Ribeiro CA, Salgado AJ, Fraga JS, **Silva NA**, Reis RL and Sousa N, 2011, The Secretome of Bone Marrow Mesenchymal Stem Cells Conditioned Media Varies with Time and Drives a Distinct Effect on Mature Neurons and Glial Cells (Primary Cultures), *Journal of Tissue Engineering and Regenerative Medicine* 5: 668–672.

Ribeiro-Samy S, **Silva NA**, Correlo VM, Fraga JS, Pinto L, Teixeira-Castro A, Almeida Leite H, Almeida A, Gimble JM, Sousa N, Salgado AJ, Reis RL, Development and Characterization of a PHB-HV-based 3D Scaffold for a Tissue Engineering and Cell-Therapy combinatorial approach for Spinal Cord Injury Regeneration, Submitted

Sousa EC, **Silva NA**, Salgado AJ, Oliveira AL, Sousa N, Reis RL, Peripheral Mineralization of a 3D Biodegradable Tubular Construct as Way to Enhance Guidance Stabilization in Spinal Cord Injury Regeneration, *Journal of Materials Science: Materials in Medicine*, 2012. Accept for publication

Cerqueira S, Oliveira J, **Silva NA**, Ribeiro-Samy S, Leite-Almeida H, Almeida A, Mano J, Sousa N, Salgado A, Reis RL. In Vitro Modulation of Microglial Cells Viability by Nanoparticle-Mediated Intracellular Release of Methylprednisolone, submitted.

Gouveia A, Martins A, Costa-Pinto A, **Silva NA**, Faria S, Sousa RA, Salgado AJ, Sousa N, Reis RL, Neves N, Osteogenic Potential of Human Wharton's Jelly Stem Cells Cultured on Hierarchical Starch-Based Fibrous Scaffold, Submitted

Book Chapters

Salgado AJ, **Silva NA**, Neves NM, Reis RL and Sousa N, 2008, "Hydrogel Based Systems for Spinal Cord Injury Regeneration", In Handbook of Natural-based Polymers for Biomedical Applications, eds. Reis RL, Neves NM, Mano JF, Gomes ME, Marques AP, Azevedo HS, Woodhead Publishing Limited, Cambridge.

International Conferences

Salgado AJ, Sousa RA, Oliveira JT, **Silva NA**, Neves NM, Reis RL and Sousa N, "Novel Tissue Engineering 3D Scaffolds for Spinal Cord Injury based on Starch/Polycaprolactone Blends: Development and Preliminary Assessment of Their Biological Performance", TERMIS-EU 2007, London, United Kingdom, September 2007 (*oral presentation*).

Fraga JS, **Silva NA**, Neves NM, Reis RL, Sousa N and Salgado AJ, Differential Effects of the Secretome of Mesenchymal Progenitors from the Umbilical Cord on the Survival and Viability of Hippocampal, Cerebellar and Cortical Primary Cultures of Neurons, 11th Meeting of the Portuguese Society for Neurosciences, Braga, Portugal, June 2009, Braga, Portugal, June 2009 (*poster presentation*).

Kim C, **Silva NA**, Sousa RA, Alves CM, Salgado AJ, Sousa N, Khang G. and Reis RL, Effect of SPCL Scaffolds on the Attachment, Proliferation and Phenotype of Olfactory Ensheathing Cells, 2nd TERMIS World Congress, Seoul, Korea (south), August 2009 (*poster presentation*).

Samy SM, **Silva NA**, Correlo V M , da Silva RMP , Fraga JS, Pinto L, Salgado AJ, Sousa N and Reis RL, Processing and Biological Evaluation of a PHB-HV 3D Scaffold

for Spinal Cord Injury Regeneration, 5th Annual International Meeting of the Portuguese Society for Stem Cells and Cellular Therapies, Guimaraes, Portugal, May 2010 (*poster presentation*).

A. Gouveia, A. Martins, A.R. Costa-Pinto, **NA Silva**, R. Reis, N. Neves, Evaluation of the Potential of Hierarchical Starch-Based Fibrous Scaffold for Bone Tissue Engineering Applications, TERMIS-EU 2010 , Galway, Ireland, June 2010 (*oral presentation*).

Teixeira FG, Carvalho MM, **Silva NA**, Neves NM, Reis RL, Sousa N, Pinto L and Salgado AJ, Transplantation of Human Umbilical Cord Perivascular Stem Cells (HUCPVCs) and their Conditioned media enhances Proliferation, Survival and Differentiation in Central Nervous System, 6th Annual International Meeting of the Portuguese Society for Stem Cells and Cellular Therapies (SPCE-TC), Cantanhede, Portugal, April 2011 (*oral presentation*).

Samy SM, **Silva NA**, Correlo VM, da Silva RMP , Fraga JS, Pinto L, Pinto LG, Castro A, Salgado AJ, Sousa N and Reis RL, Development and Characterization of a PHB-HV 3D scaffold for a Tissue Engineering and Cell-Therapy combinatorial approach for Spinal Cord Injury Regeneration, TERMIS-EU, Granada, Spain, June 2011 (*poster presentation*).

Teixeira FG, Carvalho MM, **Silva NA**, Neves NM, Reis RL, Sousa N, Pinto L and Salgado AJ, Human Umbilical Cord Perivascular Stem Cells (HUCPVCs) and their Conditioned media increase Proliferation, Survival and Differentiation in the Dentate gyrus of Adult Rat Hippocampus, TERMIS-EU, Granada, Spain, June 2011 (*oral presentation*).

INTRODUCTION TO THESIS FORMAT

This thesis is divided into four sections that include a total of eight chapters. The body of the thesis chapters is based on the series of related papers published or submitted for publication in peer-reviewed journals. Each thesis chapter is presented in the manuscript form, i.e. with the abstract, introduction, materials and methods, results, discussion and conclusions sections. A list of references is also provided as a subsection within each chapter. The contents of each chapter are summarized below.

Section I (Chapter I)

Chapter I presents a comprehensive and extensive overview on the spinal cord injury (SCI) condition. It starts covering areas from physiology and anatomy of the spinal cord, neuropathology of the SCI, current clinical options, neuronal plasticity after SCI, animal models and recovery assessment techniques, focusing the subsequent discussion on a variety of promising neuroprotective, cell-based and combinatorial therapeutic approaches that have recently moved, or are close to, clinical testing. New research directions are also proposed in the end of this chapter.

Section II (Chapter II)

Chapter II fully describes the experimental work and protocols related to the obtained results. Although each part of the work is accompanied by its specific materials and methods section, this chapter intends to condensate and assemble the relevant information on this matter.

Section III (Chapter III to VII)

These chapters describe the experimental work performed within the scope of this thesis.

Chapter III describes the development and characterization of a novel hybrid tubular scaffold aimed at SCI repair. This biphasic scaffold comprises two natural polysaccharide based biomaterials: starch/poly- ϵ -caprolactone (SPCL) and gellan gum. After the scaffold development, this chapter focuses on the physical (porosity and mechanical behavior) and biological (cytotoxicity, 3D culture and biocompatibility) characterization of the abovementioned structures.

Chapter IV reports on the importance of spine stabilization in SCI rats. The vertebral column of SCI rats was stabilized using the SPCL scaffolds developed in the previous chapter. The locomotor behavior of the rats was then analyzed by three different tests (BBB, rotarod and open field) and neuronal regeneration was assessed by histological analysis (H&E).

Chapter V presents the SPCL scaffolds as a 3D culture system. In this chapter, olfactory ensheathing cells (OECs) and Schwann cells were cultured on the SPCL fibbers. The metabolic activity, proliferation, morphology and migration were then analyzed.

Chapter VI explains the synthesis of a biologically functional gellan gum hydrogel. It describes the use of Diels-Alder click-chemistry to immobilize a fibronectin derived peptide on the gellan gum structure. The bioactivity of the novel hydrogel was tested using neural stem cells (NSCs) and confocal microscopy. Moreover, this chapter describes the positive effect of the OECs secretome on the NSCs survival and proliferation.

Chapter VII presents the final experimental study performed within the scope of this thesis. In this chapter, the secretome of OECs and adult stem cells derived from umbilical cord, adipose or bone marrow tissue was studied. The metabolic activity,

proliferation and differentiation of adult stem cells were analyzed when they were culture under the influence of the OECs secretome. The stem cells secretome effects on OECs culture were also consider.

Section IV (Chapter VIII)

This chapter contains the general conclusions of the works carried out under the scope of this thesis. Some specific remarks and future prospects are also provided.

SECTION I

CHAPTER I

Introduction - From Basics to Clinical: A Comprehensive Review on Spinal Cord Injury

CHAPTER I

From Basics to Clinical: A Comprehensive Review on Spinal Cord Injury*

Abstract

Spinal cord injury (SCI) is a devastating neurological disorder that affects thousands of individuals each year. Over the past decades enormous progress has been made in our understanding of the molecular and cellular events generated by SCI, providing insights into crucial mechanisms that contribute to tissue damage and regenerative failure of injured neurons. Current treatment options for SCI include the use of high dose methylprednisolone, surgical interventions to stabilize and decompress the spinal cord, and rehabilitative care. Nonetheless, SCI is still a harmful condition for which there is as yet no cure. However, it is no longer a matter of whether regeneration can occur. Cellular, molecular, rehabilitative training and combinatorial therapies have been shown promising results in animal models. Nevertheless, work remains to be done to ascertain whether any of these therapies can safely improve outcome after human SCI. This review provides an extensive overview of the SCI universe. It starts covering areas from physiology and anatomy of the spinal cord, neuropathology of the SCI, current clinical options, neuronal plasticity after SCI, animal models and recovery assessment techniques, focusing the subsequent discussion on a variety of promising neuroprotective, cell-based and combinatorial therapeutic approaches that have recently moved, or are close to, clinical testing.

***These chapter is based on the following publication:**

Silva NA, Sousa N, Salgado AJ and Reis RL, “From Basics to Clinical: A Comprehensive Review on Spinal Cord Injury”, Submitted

1. Introduction

The Edwin Smith papyrus, an ancient Egyptian physician textbook, has described in 1700 BC the Spinal Cord Injury (SCI) as a “ailment not to be treated” [1]. Now, almost 4000 years later, the treatment of SCI remains mainly palliative: preventing injury progression; treating spasticity, dysautonomia, and deafferentation pain syndromes; implementing bowel and bladder regimens; managing complications of sensory loss; and teaching patients how to cope with their disabilities. Fortunately, ongoing advances in neurobiology research promise to change this paradigm from palliation to cure.

The number of people in the United States who currently live with SCI is estimated to be around 253,000 with 11,000 new cases occurring each year. Causes include penetrating bullet wounds and other forms of violence (26%) and nonpenetrating lesions from vehicular accidents (38%), and sports accidents (7%), as well as falls (22%), especially in elderly persons [2]. This condition usually leads to devastating neurological deficits and disabilities that provoke not only the loss of sensory and motor capabilities (paraplegia or tetraplegia) but other common problems related with SCI as frequent infections in bladder, kidneys, bowel problems, and cardiac and respiratory dysfunctions. All of these problems have a strong impact on the physiologic, psychological and social behavior of SCI patients. For all of these reasons, is urgent to develop therapeutic strategies that can specifically target this problem.

This review intends to provide an extensive overview of the SCI universe. It will expand from the basic biology to the current state of research and clinic areas and finish with the future trends for this field.

2. Central Nervous System

Every thought, action or emotion reflects the activity of the nervous system, which is divided into the **central nervous system** (CNS) that includes the **brain** and **spinal cord**, and the **peripheral nervous system** (PNS), which includes the cranial nerves arising from the brain and the spinal nerves arising from the spinal cord.

Although exceedingly complex, nervous tissue is made up of just two principal types of cells [3, 4]:

(1) **Neurons** (Fig.1A) are the basic structural and functional units of the nervous system. Neurons vary in appearance, but all of them have just three parts: a cell body, dendrite(s), and an axon. The cell body contains the nucleus as well as other organelles. In motor neurons, the dendrites are the many short extensions that receive signals from sensory receptors or other neurons. At the dendrites, signals can result in nerve impulses that are then conducted by an axon. The axon is the portion of a neuron that conducts nerve impulses. Any long axon is also called a nerve fiber. Long axons are covered by a white myelin sheath formed from the membranes of tightly spiralled glia cells. Neurons are specialized to respond to physical and chemical stimuli, conduct impulses, and release specific chemical regulators. Through these activities, neurons perform such functions as storing memory, thinking, and regulating other organs and glands;

(2) **Glia cells** are supportive cells in the nervous system that aid the function of neurons. For example, types of glia cells found in the brain are microglia, astrocytes, and oligodendrocytes. **Microglia** (Fig.1B) cells are the immune mediators of the CNS, possessing a macrophage like behaviour, phagocytosing dead tissue and cells, as well

foreign agents. **Astrocytes** (Fig.1C) are the most abundant glial cell type. They are responsible for mediating the synaptic transmission, provide nutrients for the neurons and simultaneously they make part of the blood brain barrier (BBB), which can be look as a filter against foreign agents that intend to enter the CNS. **Oligodendrocytes** (Fig.1D) are the cells involved on the production of the myelin sheath, which encases the neurons. Therefore they are of the utmost importance within the CNS, as the loss of myelinization leads to extensive damage within the latter, as it happens during SCI. With similar function, the Schwann cells are the type of glia that produces the myelin layer in the PNS. The areas of the nervous system that are rich in myelinated fibers are known as white matter while the areas containing the cell bodies and demyelinated neurons are known as grey matter [5, 6].

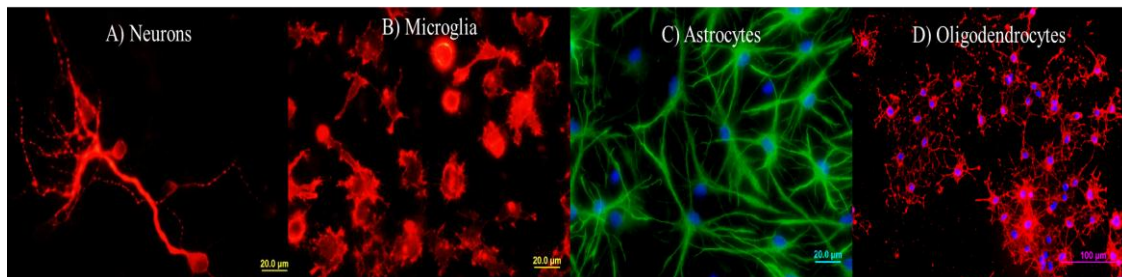


Figure I.1. Immunocytochemistry images of CNS cells. (A) MAP-2 positive neuron. (B) CD11B positive microglia. (C) GFAP positive astrocytes. (D) O4 positive oligodendrocytes.

3. Physiology and Anatomy of the Spinal Cord

The spinal cord provides a means of communication between the brain and the peripheral nerves that leave the cord. Besides that, it is also able to produce reflexes, called the

spinal reflex. Specific nerve pathways allow for reflexive movements rather than those initiated voluntarily by the brain, such as coughing, sneezing and vomiting.

The spinal cord extends from the base of the brain (in medulla oblongata) through the foramen magnum of the skull to the first lumbar vertebra. The cord only extends to the L1 vertebra in human, or L3 in the case of rats, because the vertebral column grows faster than the spinal cord. For this reason, and below the cervical levels, the spinal nerves run increasingly obliquely downwards to their intervertebral foramina. The spinal cord is protected by the vertebral column, which is composed of individual vertebrae. It is also protected by three membranes of connective tissue called meninges. From the outside in, the meninges are the *dura mater*, *arachnoid mater* and *pia mater*. Finally and helping to protect the spinal cord too, as well with others function, there are the subarachnoid space (between arachnoid and pia) filled with cerebrospinal fluid and the epidural space (between dura and periosteum) filled with loose fibrous and adipose connective tissues [3, 5, 7].

Unlike the brain, in which the gray matter forms a cortex over white matter, the **gray matter** of the spinal cord is located centrally, surrounded by **white matter**. The central gray matter is arranged in the form of a butterfly. The projections of the gray matter within the spinal cord are called horns. The two dorsally projecting arms are called the **dorsal horns**, and the two ventrally projecting arms are called the **ventral horns** (also called posterior and anterior horns, respectively). The gray matter is made up of interneurons, the cell bodies and dendrites of efferent neurons, the entering fibers of afferent neurons, and glial cells. The surround white matter (except when the dorsal horns

touch the margins of the spinal cord) is composed mostly of groups of myelinated axons of interneurons. These groups of axons, called fiber tracts or pathways, run longitudinally through the cord, some descending to relay information from the brain to the periphery, others are ascending to transmit information to the brain (Fig.2).

The names of the **ascending tracts** usually start with the prefix spino- and end with the name of the brain region where the spinal cord fibers first synapse. The anterior spinothalamic tract, for instance, carries impulses conveying the sense of touch and pressure, and synapses in the thalamus. From there it is relayed to the cerebral cortex. The names of **descending motor tracts**, conversely, begin with a prefix denoting the brain region that gives rise to the fibers and end with the suffix -spinal. The lateral corticospinal tracts, for instance, begin in the cerebral cortex and descend the spinal cord. More information about the most important tracts that run in the spinal cord can be found in the Watson, Paxinos and Kayalioglu book [8].

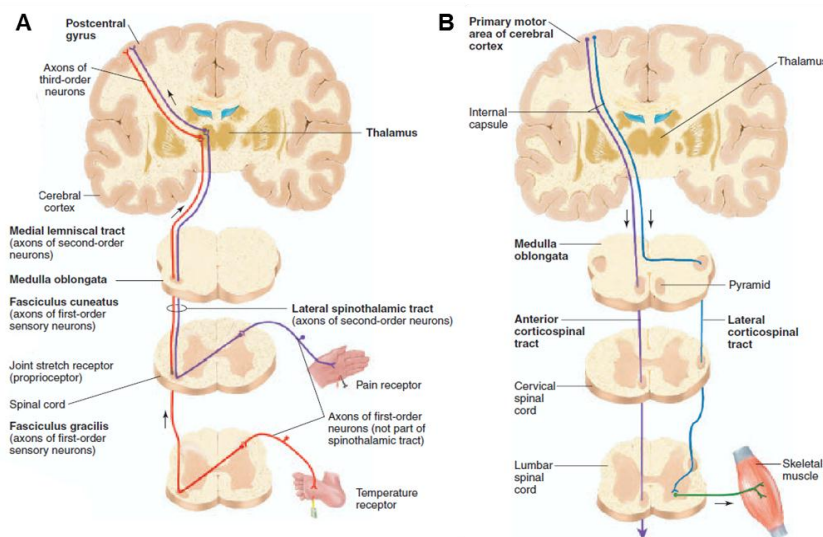


Figure I.2. Ascending and descending spinal tracts. (A) Lateral spinothalamic tract carrying sensorial information from peripheral organs to the brain. (B) Pyramidal corticospinal tracts carrying motor information from cortex to peripheral organs. Adapted from reference 3.

The above mentioned fiber tracts are crucial in the communication between spinal cord and brain. Groups of **afferent** (sensory) **fibbers** that enter the spinal cord from the peripheral nerves enter on the dorsal side of the cord via the dorsal roots. Small bumps on the dorsal roots, the dorsal root ganglia, contain the cell bodies of the afferent neurons. The axons of **efferent** (motor) neurons leave the spinal cord on the ventral side via the ventral roots. Near the cord, the dorsal and ventral roots from the same level combine to form a **spinal nerve**, one on each side of the spinal cord. The spinal nerves are designated

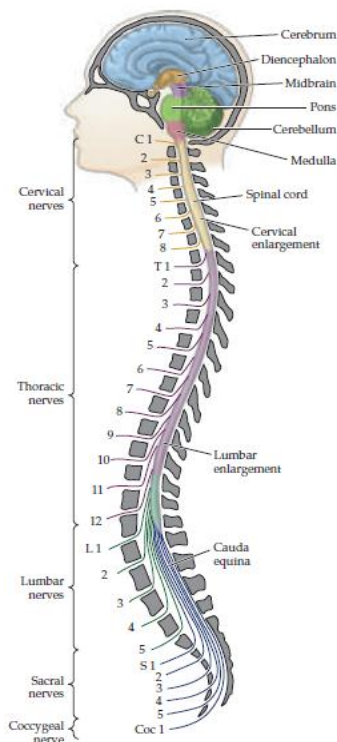


Figure I.3. Set of the 31 spinal nerves. Adapted from reference 4.

by the five vertebral levels: from which they exit: cervical, thoracic, lumbar, sacral and coccygeal. The human spinal cord comprises **31 spinal nerves** (Fig. 3): **8 cervical nerves** (C) – control the muscles and glands and receive sensory input from the neck, shoulder, arm and hand; **12 thoracic nerves** (T) - associated with the chest and abdominal walls; **5 lumbar nerves** (L) - associated with the hip and leg; **5 sacral nerves** (S) - associated with the genitals and lower digestive tract; and **1 coccygeal nerve** (Coc) – supply the skin over the coccyx. Although the spinal cord of the rat comprises 34 spinal nerves - 8 cervical, 13 thoracic, 6 lumbar, 4 sacral and 3 coccygeal – the general associated functions are similar.

4. Neuropathology of Spinal Cord Injury

4.1. *From acute to chronic injury*

When the spinal cord is lacerated or macerated by a sharp penetrating force, contused or compressed by a blunt force (most common [9]), or infarcted by a vascular insult, it begins a neurological damage in the spinal cord that is normally called “**primary injury**”. Based on imaging and histology of injured human spinal cords, traumatic SCI was classified by Bunge and colleagues as: contusion with cavity formation; massive compression; or laceration [10, 11]. The mechanical injury leads to a cascade of biological events, describe as “**secondary injury**”, which occurs over the time course of minutes to weeks and provokes further neurological damage. Finally, and occurring over a time course of days to years, there is the onset of **chronic phase**, that leads to neurological impairments in both orthograde and retrograde directions, including brain regions [12, 13].

The understanding of the biochemical and cellular events that compose the secondary and chronic phase is of utmost importance, since it could provide significant information that might lead to promises therapies that minimize the extension of the lesion and improve the regeneration. In 1911, Alfred Allen [14] was the first suggesting that secondary events happen after spinal cord injury. He reported neurological dependent functions improvements after the removal of inflammatory fluid in spinal cord injury of dogs. Therefore, Dr. Allen theorized that it was a noxious agent present in the hemorrhagic fluid that might cause further damage to the spinal cord [15]. Following him, several

authors postulate numerous biochemical mechanisms that elucidate the progressive post-traumatic damage of spinal cord tissue. These secondary events consist of:

- **Vascular changes** - includes hemorrhage, vasospasm, thrombosis, loss of autoregulation, breakdown of blood brain barrier and infiltration of inflammatory cells. This leads to edema, necrosis and ischemia [16-18];

- **Free radical formation and lipid peroxidation** - causes oxidative death in spinal cord neurons [19] and reduce the spinal cord blood flow leading again to edema and inflammatory response [20, 21]. Moreover, it has been shown that lipid peroxidation affects the ATPase activity [22] as well cause cytoskeleton degradation [23];

- **Disruption of ionic balance of K^+ , Na^+ , Ca^{2+}** - leads to depolarization of cells membranes, ATPase failure and increase of intracellular Ca^{2+} [24]. All of these events increase cell death;

- **Glutamate excitotoxicity** - after the SCI there is an increased release of extracellular amino acids, namely glutamate, that induce excessive activation of glutamate receptors leading to further neuronal cell death [25-28];

- **Apoptosis** - the programmable mechanism of cell death occurs after SCI and involves reactive gliosis that includes increased expression of glial fibrillary acidic protein (GFAP) and astrocyte proliferation. Moreover, demyelination of tracts away from the injury, appear to be due in part to apoptosis [29].

- **Inflammatory response** - following neural trauma the resident microglia become activated, while rapidly infiltrating leukocytes start to release an increased amount of cytokines and reactive oxygen species (ROS) [30], which will allow higher extravasation of leukocytes and further tissue damage [31-33]. Nevertheless, some studies

demonstrated that inflammation also plays an important role in neural tissue repair [34]. The challenge for researchers is to learn how to control the interactions between the nervous and immune systems to minimize delayed neurodegeneration while promoting axonal and regeneration. For example, the expression of the proinflammatory cytokine $\text{TNF-}\alpha$ is upregulated after a neural damage leading to oligodendrocytes death and white matter injury [35, 36]. However, White and colleagues [37] demonstrated that $\text{TGF-}\alpha$ infusion preserve the supportive and survival effects that the astrocytes have under the neurons, and altered astrocytes activation.

Finally, the **chronic phase** comprises events like white matter demyelination, gray matter

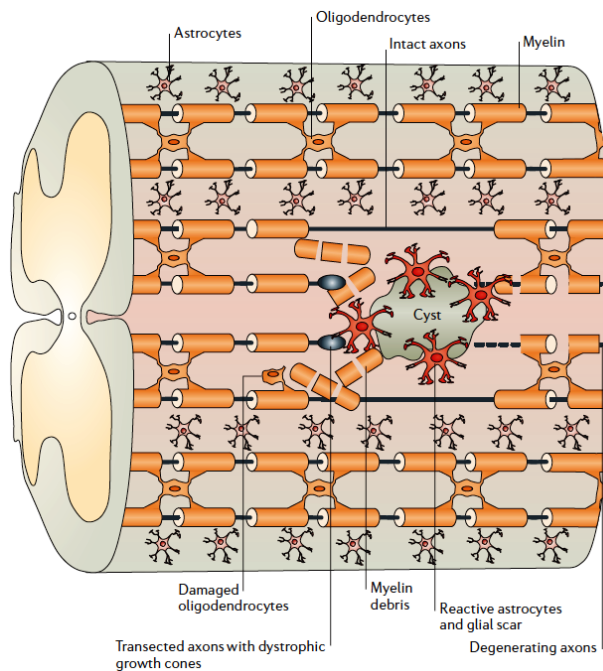


Figure I.4. Schematic representation of the SCI site. Adapted from reference 13.

dissolution, connective tissue deposition and reactive gliosis that lead to glial scar formation. The glial scar works like a physical barrier, do not allowing axons to grown across it. It is formed predominantly by reactive astrocytes, microglia/ macrophages, and extracellular matrix molecules, especially chondroitin sulfate proteoglycans [38-40]. Moreover, in approximately 25% of SCI patients the glial scar surrounds a cystic cavity

that progressively expands leading to a condition called syringomyelia. Finally the input

changes in inhibitory and excitatory neurotransmitters and in many cases the permanent neuron hyperexcitability, results in chronic pain syndromes and consequently mood disorders such as depression [41, 42].

4.2. Molecules with growth-inhibitory effects in CNS

The external environment greatly influences the intrinsic growth ability of an injured neuron. It is known that during the injury phases several molecules display inhibitory effects in neuron regeneration. The most studied group is the **myelin-associated molecules**. After the injury, the disruption myelinated axons as well as oligodendrocytes dead, leads to the release of myelin debris. Among these, the **Nogo** protein is the most “famous”. Nogo is a membrane protein expressed by oligodendrocytes and some neurons, and causes growth inhibition and growth cone collapse when binding to its receptor in neurons membrane [43, 44]. Other molecule of this group is the **myelin-associate glycoprotein (MAG)**. MAG is one of the inhibitors of white matter regeneration and there are several studies, both in vitro and in vivo, that show the inhibitory capability of MAG [45-47]. However the mechanism of neural growth inhibition is not fully understood. Finally, **Tenascin R** is an inhibitory molecule present in extracellular matrix of CNS, which is produced by oligodendrocytes and is upregulated after injury. Tenascin R interacts with the axonal receptor F3/11 inhibiting its growth. Tenascin R also interacts with several proteoglycans implicated in growth inhibition [48-50].

The **proteoglycans** are the second major group of molecules that present an inhibitory profile after a CNS injury. These molecules consist of a protein core linked by four sugar moieties to a sulphated glycosaminoglycan (GAG) chain that contains repeating disaccharide units [51]. Proteoglycans are mainly expressed by astrocytes, oligodendrocyte precursors and meningeal cells and are deeply involved in the glial scar formation [52]. There are four known classes of proteoglycans: (i) heparan sulphate proteoglycans (HSPGs), (ii) dermatan sulphate proteoglycans (DSPGs), (iii) keratan sulphate proteoglycans (KSPGs) and (iv) **chondroitin sulphate proteoglycans (CSPGs)** [51]. The last ones are the most important and studied. The CSPGs forms a relatively large family, which includes aggrecan, brevican, neurocan, NG2, phosphacan and versican. There are several evidences about the axon regeneration inhibition profile of CSPGs. It was demonstrated that CSPGs expression increase after and around the CNS injury site [53-55], and more importantly, Davids *et al.* [56] show that sensory neurons are capable to repair their axons through the white matter, but the regeneration stops when are CSPGs deposition. More information about CSPGs and glial scar can be found in Fawcett and Silver reviews [52, 57].

It is important to note that the role of glial scar is very complex. Despite of all studies demonstrating its detrimental effects [58, 59] some others studies have shown a beneficial role of the glial scar during the acute phase (1–2 weeks) after spinal cord injury. Elimination of reactive astrocytes or preventing their migration and scar formation after injury resulted in a failure of blood-brain barrier repair accompanied by massive inflammatory cell infiltration and increased loss of neurons and oligodendrocytes with a

consequent worse functional outcome [60, 61]. Thus, an acute astrocytic response appears important to limit and restrain the inflammatory response, but this may be at the expense of reduced axonal regrowth.

5. Neuronal Plasticity after Spinal Cord Injury

Derived from the Greek word *plassein*, plasticity refers to the ability of being molded or altered. The CNS is highly malleable, experiencing plasticity over its lifetime. The process of learning, skill acquisition and specially the response to an injury provokes neuronal reorganization, synaptic rearrangements, changes in the neuronal activation pattern and intact or lesioned axon collateral sprouting. Reports in both animal models and human patients revealed that some regions from brain undergo plasticity after a central or peripheral injury. Kaas and colleagues, for instance, described that the cortical territories controlling intact body parts of monkeys with long-standing amputations tend to enlarge and invade those that have lost their peripheral targets [62]. Similar cortex reorganization was also observed in humans with a SCI [63-65]. Moreover, Jain *et al.* observed that complete cervical spinal cord lesions deactivated the forelimb, trunk and hindlimb representations in the somatosensory cortex [66]. Additionally, Cramer *et al.*, using functional magnetic resonance imaging (fMRI), described that the sensorimotor cortex of chronic, complete SCI patients, presented a volume of activation much reduced than controls [12]. The cortex is not the only region affected by a SCI. Subcortical structures such as the red nucleus and the cuneate nuclei of the brain-stem also experience plasticity. For instance, Lawrence *et al.* previously described that a novel pathway is formed between the left and the right red nucleus in monkeys subjected to

unilateral corticospinal tract (CST) lesions. The axonal sprouting between both red nucleus was responsible of the partial recovery of the voluntary control of arm and foot [67, 68]. The axonal sprouting of sensory fibers in the cuneate nuclei after SCI has also been described. For example, Kass and colleagues demonstrated that incomplete SCI lesions led to sprouting of spared forelimb primary afferents in the cuneate nuclei of the brain stem and this led to the reactivation of forelimb representations on the somatosensory cortex [66, 69].

Concerning the spinal cord tissue, several studies were able to confirm that plasticity takes place after injury. This was first suggested by the observation that paralysed cats with complete spinal cord transection could be trained to walk on a treadmill [70, 71]. In the first days after SCI, cats could not support their body and could not initiate spontaneous rhythmic movements. However, after one month, the injured cats were able to perform coordinated movements. These studies also pointed out the fact that plasticity is significantly influenced by physical activity. Trained injured cats showed functional recovery three times greater than untrained animals [71]. The finding that the spinal cord had plastic properties led to novel rehabilitation strategies for SCI in humans. Patients with incomplete SCI achieved significant functional benefits by daily training on a moving treadmill [72]. Nowadays, locomotion training is becoming routine for incomplete SCI patients all over the world.

Studies in animal models further elucidated us about the events that are behind the functional recovery after an incomplete SCI. The spinal cord is capable to adapt by mechanisms involving spared long-tract axon collateral sprouting [73], sprouting of

spinal interneurons [74], adaptations of the motoneurons caudal to the injury [75], and by functional redundancy [76].

The specific biological mechanisms behind the neuronal plasticity are still fairly known. The components of the secondary insult arising from the SCI may play an important role in the plasticity observed. The damaged BBB and the immune response after the SCI expose the nervous tissue to a high concentration of cytokines and growth factors that induce changes at the cellular level of the spinal cord. Moreover, the mechanism with strongest evidence in mediating changes in plasticity is the removal of GABAergic inhibition in excitatory synapses. GABA is the most important inhibitory neurotransmitter in the brain and GABAergic neurons constitute 25~30% of the neuronal population in the motor cortex [77]. Several studies have shown that modulation of GABAergic inhibition plays a significant role in cortical plasticity [78, 79]. Moreover, *Roi et al.* demonstrated that the GABAergic intracortical inhibitory circuit is reduced in SCI patients [80]. These findings may help to explain the cortical plasticity observed after a SCI.

It is now clear and well accepted that plasticity occurs in the mammalian CNS in response to injury. However, it is also clear that not all the plasticity is beneficial. Patients with complete SCI exhibited several abnormal features of brain activation, which some studies suggest a partial loss of motor cortex function. In this sense, brain events that normally occur during voluntary actions are limited after SCI and may also contribute to the inability of patients to perform movements. For therapies that aim to restore motor function after SCI, alterations in the brain functions mean that the initiation of movements might not occur normally. Therefore, addressing these changes may be

important to implement a successful therapy in complete SCI chronic patients. A tremendous number of questions still need to be addressed in order to harness plasticity, such as whether harmful plasticity can be prevented at the same time when beneficial changes are encouraged.

6. Highlights of Clinic State for Spinal Cord Injury

To date, there are no efficient and trustworthy clinic treatments available for SCI patients. The usual surgical procedures made by physicians are the **stabilization** and decompression of spinal cord combined with a high dose methylprednisolone (MP) therapy [81]. However, surgery and MP approaches are deeply involved in controversial since there are no consensuses about the true beneficial effects in both topics.

6.1. *Surgical Interventions*

There are strong evidences from animal models that **decompression** of spinal cord improves recovery and minimize the secondary injury after SCI [82-85]. On the other hand the outcome from human trials is not so clear, as there are several reports that have argued against surgery, especially early intervention in critically ill patients [86-88]. The question about the optimal timing of surgical interventions (or even the intervention) has generated considerable debate and remains unanswered for the entire spine. However, the number of studies showing that early decompression have a neuroprotective effect in humans is much higher than the studies that show no effects or negative impact [89]. Nevertheless, the benefits of early intervention are yet to be fully demonstrated. It is

important to be aware about the complexity and the individuality of SCI. This can explain why there are no conclusive data about surgery as a valid practice option. In this sense, prospective, randomized and multicenter clinical trials are needed in the future in order to gain valuable data.

6.2. *Pharmacological Interventions*

The delivery of a pharmacological agent shortly after acute SCI that might improve neurological function and/or support neurological recovery has long been explored. A variety of promising drugs have been tested in animal models, but few have had potential application to human SCI patients. **Methylprednisolone** is the most prescribe agent in clinical practice, however, is also the most controversial. MP is a corticosteroid identified as being able of inhibit the lipid peroxidation, maintain the blood-spinal cord barrier, enhance spinal cord blood flow, inhibit endorphin release, limit the inflammatory response and scavenge damaging free radicals [90-92]. These pre-clinical findings led, in 1979, to the initiation of a multicenter, randomized, double-blinded clinical trial named National Acute Spinal Cord Injury Study (NASCIS). The analysis of 330 patients revealed no differences in neurological recovery, motor and sensory function, between groups either at 6 weeks or 6 months after the injury [93]. Further animal studies suggest that the MP dose used in NASCIS I was not sufficient to elicit neuroprotection [94, 95]. Thus, a multicenter NASCIS II trial was initiated in 1985 using a much higher dose of MP. Five years later and after analysed 487 patients, Bracken and his team reported that the administration of high dose of MP within 8 hours after injury was associated with a significant improvement in motor and sensory function at the 6-month follow up

compared with patients receiving placebo, naloxone or MP at later times [96]. Despite of these results the NASCIS II trial has not been universally accepted. Several methodological, scientific and statistical issues were criticized [97-101]. For instance, only upon stratification of the data was possible to obtain statistical differences. These originate concerns about the small sample population size for the groups showing beneficial effects. Moreover, there were no functional outcome measures defined to assess whether the statistical improvements noted with MP were correlated with clinical significance. These critics led to the development of a third NASCIS. The study initiated in 1991 and published in 1997 involved 499 patients and additional functional outcome was measure. Namely, the Functional Independence Measure (FIM) was used to assess self-care, mobility, locomotion, sphincter control, communication and social cognition of acute SCI patients. The authors reported that no benefit was associated with extending MP administration beyond 24h if patients had received MP within 3h of injury. Although, patients that received MP between 3 and 8h after the injury demonstrated improvements in motor capabilities if drug administration was continued for 48h in comparison to 24h group [102]. These differences were observed only at the 6 weeks and 6 months follow-up and no statistically significant differences were observed for FIM scale at 1 year [103]. After the NASCIS II and III the administration of MP to acute SCI patients was a common practice among physicians. However, the intense criticisms to both clinical trials associated with the fact that all NASCIS studies reported a statistically significant increase in wound infections, gastrointestinal hemorrhages, sepsis, pulmonary embolism, severe pneumonia and death culminated with the following opinion from the American Association of Neurological Surgeons/Congress of Neurological Surgeons

(AANS/CNS): “Methylprednisolone for either 24 or 48 hours is recommended as an option in the treatment of patients with acute spinal cord injuries that should be undertaken only with the knowledge that the evidence suggesting harmful side effects is more consistent than any suggestion of clinical benefit” [104]. Hulbert *et al.* reported that after the recommendations of AANS/CNS there has been a complete reversal in Canadian clinical practice patterns of MP administration for SCI. The authors showed that in just five years the number of surgeons that prescribed MP changed from 76% to just 24% [105].

In order to avoid adverse side effects derived by systemic high dose injections of MP, directed and sustained delivery of methylprednisolone by means of an adequate drug delivery system, for instance a nanoparticle-based system, is under evaluation [106, 107]. Although much more pre-clinical data are needed, these safer and more efficient routes of delivering MP to neuronal cells after a SCI might be extremely beneficial to protect and regenerate the nervous tissue.

Other pharmacological agents, such as monosialotetrahexosylganglioside (GM-1), thyrotropin-releasing hormone (TRH), gacyclidine, naloxone and nimodipine, have been subjected to investigation in large multicenter prospective randomized controlled clinical trials [108]. However, none of them have demonstrated clinical beneficial outcomes in SCI patients. In this sense, the search for the pharmacological agent with the ability to improve neurological function is still open. Many promising therapies are currently under pre-clinical investigation with the potential of entering in clinical trials in the future (see section 9.2 of the present chapter).

7. Animal Models in SCI Research

The use of animal models is critical for development of experimental therapies that are aimed to repair the injured spinal cord. Animals such as cats, dogs, swine, rats, mice and non-human primates are being used as SCI models [29, 109-112]. Rat models are by far the most commonly used, mainly due to their cost, accessibility, easy to care for, and because of the existence of well-established functional analysis techniques. Mice are also widely used, essentially because of our ability to manipulate their genome and in this way dissect complex molecular events [113]. Non-human primates experiments are very limited fundamentally due to high costs and ethical reasons. However, to prove safety and efficacy prior human experiments may be essential the use, in a small scale, of non-human primates.

In experimental animal models, three general classes of injury are frequently used: transection, contusion and compression [114]. Different types of injury address different questions, and therefore each type own advantages and disadvantages.

The **transection** model requires the opening of the dura and the segmentation of some or all spinal cord with a sharp instrument [114]. The spinal cord can be transected and left in place or a small section can be removed, normally, for implantation of a specific device. Unilateral hemisection is sometimes preferred than full transection because the integrity and function of one side of the cord is preserved. This is usually sufficient to maintain bladder and bowel function which results in less post-operative care and less animal death. The transection model provides valuable information in strategies that target axon

regeneration and may be the most appropriate models for the implantation of specific devices. However, SCI such as produced by transection is rarely seen in human patients. In consequence, many researches use the compression and contusion injury models.

The **contusion** model is induced by hitting the exposed spinal cord (without disruption of the dura) with a blunt contusion force. This model is often obtained using a computer-controlled impactor. This equipment consists of an animal trap device that reproducibly delivers a weight (in the case of the New York University impactor [115]) or a solenoid (Ohio State University impactor [116]) to the spinal cord, with a computer monitoring the impact. These devices allow the regulation of biomechanics events such as impulse, velocity, power and energy, thus permitting a superior control of the injury degree. Moreover, the contusion type of injury better mimic the lesions seen in human than the transection models [117]. However, in this model it is difficult to distinguish between the spare and regenerated tissue.

The **compression** model was introduced by Rivlin and Tator in 1978. The injury is induced by compressing the spinal cord with a modified aneurysm clip [118]. Similar injuries using forceps or a compression balloon have also been described [119, 120]. Similar to impactor models, the compression model can create different degrees of SCI by adjustment of the compression strength, the compression time, or both [121]. The compression model also mimics the neuropathology over time observed in humans. It begins with an early phase of spreading hemorrhagic necrosis and edema, progressing to a phase of partial repair and reorganization, and ending in a chronic phase with the

central cyst formation, atrophy and glial scar. Mainly used by Fehlings and Tator labs, the clip compression model has provided valuable knowledge about pathophysiology of SCI [122], timing of decompression [123] and neuroprotective agents [124]. The difficulty in verify anatomical axonal regeneration is also the major disadvantage of this model.

8. Recovery Assessment

An accurate and reliable evaluation of functional recovery after SCI is an essential step for those attempting the repair of the spinal cord and/or to study the spontaneous functional improvements over time. Behavioral analysis (motor and sensory), histological staining and electrophysiology are the mainly used techniques to determine functional and anatomical recovery. The proper selection of anatomic, neurophysiologic and functional tests before beginning an animal study is crucial for its success.

8.1. Behavior Evaluation

The key element of any behavioral test is the ability to produce reliable, reproducible and worthwhile data [125]. Behavior evaluation in SCI animals are currently being used to: (i) correlate the degree of functional deficits with lesion severity, location and duration [76, 126, 127]; (ii) to document the extent of recovery after SCI with or without therapeutic interventions [46, 128]; and/or (iii) to identify the integrity of specific motor and sensory systems [129, 130]. The behavior tests used in SCI are commonly classified as motor or sensory tests; however, since some could fall in more than one category,

Muir *et al.* classified them according to the type of data collected. The categories included: (i) endpoint measures, in which behavior is scored according to some goal to be reached (e.g., horizontal ladder walking test); (ii) kinematic measures, which can range from qualitative description of movements to continuous quantitative measurements (e.g., BBB scale); and (iii) kinetic measures, which quantify, for instance, the force produced by the limbs (e.g., limb grip strength test).

The most commonly test used to assess motor function after a SCI is the **BBB locomotor scale**. The BBB is a 21 point scale designed in 1995 by Basso, Beattie, and Bresnahan to assess recovery of hindlimb function after thoracic spinal injury [126]. Each point in the score represents a specific set of characteristics demonstrated by the animal during spontaneous open field locomotion. The scale range goes from 0 (no apparent movements) to 21 (normal movement, coordinated gait with parallel paw placement) being scores from 0 to 7 related with recovery of isolated movements in the hip, knee and ankle joints; from 8 to 13 associated with return of paw placement and coordinated movements with the forelimbs; and from 14 to 21 related with the recovery of toe clearance, trunk stability, and predominant paw and tail position. The BBB scale is widely used mainly because the test produce reliable, reproducible and worthwhile data. Moreover, the BBB scale owns more advantages: (i) it is easy to learn and perform; (ii) provides a meaningful measure of recovery of a clinically relevant behavior (locomotion); and (iii) animal training is not a requirement. The subjectivity of the test is the main limitation of the BBB [131, 132]. However, in order to decrease its subjectivity the test should be blind and performed by two independent researchers. Moreover, the

BBB scale cannot be used as a recovery assessment tool for all the therapeutic approaches used in SCI. Since the lower part of the scale concerns about gross aspects of locomotion, while the upper part of the scale includes discrete movement aspects, recovery assessment of a treatment that only improves toe clearance or trunk stability without having any impact on forelimb-hindlimb coordination cannot be done by using the BBB scale. In order to solve this problem and improve the sensitivity of the BBB scale a sub-scoring system was proposed [128, 133]. In the **BBB sub-scoring** scale each of the behavioral attributes is scored independently and then added them together to yield a single score. In this sense, using the sub-scoring system is possible to discriminate between gross and fine recovery.

Another commonly used test to evaluate locomotion recovery after spinal cord injury is the **horizontal ladder walking test** [134]. In this test, animals walk along a horizontal ladder with variable rung spacing. Crossing the ladder requires an accurately placement of animals' limbs on the bars. Moreover, to prevent the animals from learning the pattern and anticipating the position of the rungs, the distances between metal rungs are irregular and the pattern differs, depending on whether the animal is walking from the left or the right side. The missteps frequency is calculated as the number of foot slips by each limb divided by the total number of steps [135]. This test is simple, cheap and uncovers discrete changes in motor recovery; however, it cannot be used in complete paralyzed animals and requires training.

An exciting method to evaluate locomotor behavior is the **three dimensional analysis** of musculoskeletal movement of animals. This biomechanical calculation usually involves the use of several video cameras (or one video camera and an specific arrangement of

mirrors [136]) oriented at 45 and 135° bilaterally with respect to the direction of locomotion and reflective markers attached to the animal skin at the spine, iliac crest,

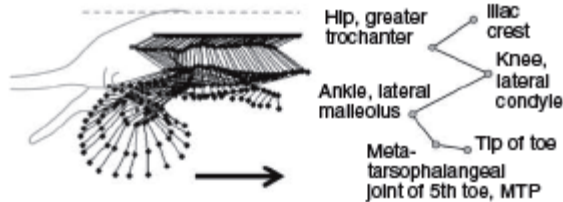


Figure I.5. Three dimensional analyses of musculoskeletal movements. Adapted from reference 136.

greater trochanter, lateral condyle, lateral malleolus, the distal end of the fifth metatarsal, and the lateral surface of the fifth metatarsal [136, 137]. With

this method is possible to obtain precise geometric locomotor profiles of the animals (Fig.5) and, with it, detect

fine motor improvements that otherwise would be impossible to distinguish. Moreover, it was previously demonstrated that it is possible to obtain animal locomotor profiles during other behavioral tests. Zörner *et al.* acquired locomotor profiles of rats and mice while animals were walking in a horizontal ladder, swimming, wadding or just walking in a plain surface [136]. In this way it was possible to study four different types of locomotion in SCI animals in a precise manner. Despite all these advantages, the three dimension analyses are expensive, time consuming, lacks standardization and sometimes data obtained from these approaches can be difficult to interpret. Nevertheless, profiling locomotion is a powerful tool to assess recovery in SCI animals.

The assessment of sensory function in SCI rats is usually done by using the hot plate and Von Frey filaments tests. The **hot plate** test measures the hind paw withdrawal latency from a radiant heat source. Animals are placed inside a plastic cage with a hot source below the hindlimbs and the test is finalized when rats lick their paw. Depending of the

protocol, rats which show no reaction to the hot source are removed after 35-60s to avoid paw injury [138, 139]. Additionally to the hot plate measurements, the **Von Frey filaments** are often used to evaluate if animals experience mechanical allodynia. Allodynia is a type of neuropathic pain characterized by an increased sensitivity to innocuous stimuli. The filaments (1-300g) are sequentially applied to the plantar surface of the fore or hindlimbs, with a pressure that causes a slight bend of the filament, until the withdrawal response is obtained. A positive reaction occurs when the paw is quickly removed away from the filament. This response may be associated with vocalization, flinching and/or abnormal aggressive behavior [140]. It is important to note that sensory tests are usually difficult to interpret as they virtually all depend on a motor end point that could of course be compromised by the SCI.

In this review we only focused on the tests most commonly found in literature. More information about behavioral testing in SCI animals can be obtained in Sedý review [141].

8.2. *Anatomic analyses*

The landmark work of Falck and colleagues in the 1960s marked the beginning of a new era in neuroanatomy. The development of methods to identify biogenic amines in tissue sections made possible the identification of neuronal population according to the neurotransmitter(s) they utilized [142, 143]. Subsequent experiments demonstrated that was possible to identify not just the biogenic amines but a wide range of conventional neurotransmitters, peptides, enzymes, and others proteins [144]. These techniques are now known as **immunohistochemistry** and provided valuable knowledge about

anatomic changes, pathophysiology, neuronal regeneration, and plasticity of the spinal cord tissue after suffer a lesion. Examples of biomarkers usually employed in SCI-related experiments can be found in Table 1. The selection of appropriated markers depend of the hypothesis that is been tested. For example, approaches aimed at SCI repair normally utilize markers that allow the observation of axon regeneration such as growth associated protein 43 (GAP 43), neurofilament (NF) for axons cytoskeleton, or/and the calculus of the amount of serotonergic neurons (5-HT). Alternatively, the experimental work aiming on the understanding of the SCI pathophysiology may use others markers such as the nogo receptor IN-1, the glial scar constituent CSPG or the GFAP for astrogliosis. The choice of appropriated biomarkers is therefore crucial for the successful anatomic evaluation of the spinal cord tissue.

Another helpful technique for identifying axons that are injured or regenerating is the employment of axonal **tracers**. Neurons can specifically internalize macromolecules and transfer them to others neurons of the same pathway. By doing that is possible to identify entire neuronal tracts, from the motor cortex to the end of the spinal cord or *vice versa*. Upon internalization the tracers can be transported in either an anterograde or retrograde fashion. Antereograde tracers are transported from the cell bodies to the axons, while retrograde tracers are transported from axons to cell bodies. It is common practice to apply a tracer distally to the injury and then evaluated its presence in the cell bodies proximally. For instance, if the spinal cord is full transected at T5 and a retrograde tracer is injected at T11, the presence of the tracer above the T5 section implies that regeneration across the lesion occurred. Examples of retrograde tracers generally employed in SCI experiments are the Fluoro-Gold, Fast-Blue, leucoagglutinin (PHA-L)

and the fluoro-emerald [145, 146]. Alternatively, anterograde tracers normally employed are the cholera toxin B subunit (CTB), the enzyme horseradish peroxidase conjugated to wheat germ agglutinin (WGA-HRP) and, the most commonly found in literature, the biotinylated dextran amine (BDA) [145-149]. It is important to note that, this technique can be misleading if the track is incompletely cut and axons have been spared, or if excessive tracer is used proximally to lesion.

Table I.1. Most popular biomarkers employed in SCI experiments.

Marker	Identifies:	Information about:	References
GAP 43	Growing neurons	Axonal regeneration	[150]
NF	Neurofilament peptide	Axonal organization	[151]
B-tubulin III / TUJ-1	Neurons	Neuronal organization	[152]
MAP-2	Neurons	Neuronal organization	[152]
BLBP	Neural progenitor	Neurogenesis	[153]
CGRP	C-Fibers axons	Neuropathic pain	[151]
PV	Parvalbumin neurons	Sensory neurons	[151]
DexTR	Sensory axons	Ascending pathways	[154]
5-HT	Serotonergic neurons	Neuronal organization	[151]
DBH	Dopamine neurons	Neuronal organization	[146]
NK1-R	Substance P	Neuronal survival	[155]
c-Fos	Neurons	Activated neurons	[149]
OX42	Microglia	Inflammation	[156]
PDGF- α R	Progenitores oligodendrocytes	Glial scar/repair	[152]
APC	Mature oligodendrocytes	Cell organization/myelin	[152]
MBP	Myelin sheath	Mature oligodendrocytes	[157]
GFAP	Astrocytes	Glial scar/ astrogliosis	[148]
p75	Schwann cells	Invading Schwann cells	[152]

Table I.1. Most popular biomarkers employed in SCI experiments. (continued)

Marker	Identifies:	Information about:	References
P0	Schwann cells	Invading Schwann cells	[152]
Active caspase 3	Apoptotic cells	Cell death	[158]
TrK A, B or C	Tyrosine kinases receptors	NGF, BDNF or NT3/4	[159]
IN-1 and NEP1-40	Nogo receptor	Inhibitory molecule Nogo	[147]
anti-CSPGs	CSPG	Glia scar	[152]
Neurocan	CSPG	Glia scar	[37]
C4S	Chondroitin	CSPG GAG digestion	[152]
MMP-2	Matrix metalloproteinases	Glial scar degradation	[160]
Laminin B2	Laminin	Permissive ECM	[37]

8.3. Electrophysiology

Electrophysiology techniques provide direct and precise measurements of muscle activation, reflex latency, reflex responses, and transmission of both somatosensory-evoked potentials and motor-evoked potentials. By doing so, neurophysiologic testing has been useful for evaluating experimental therapies for spinal cord injuries [161-164]. The technique is based on the stimulation of a specific neuronal track on one point, transmission of the evoked potential through the injured area, and impulse recording on the other point of the track. In this way is possible to obtain valuable information about injured and regenerating neuronal pathways. Electrophysiology can be useful for *in vivo* and also *in vitro* experiments. For instance, Fehlings and coworkers have provided significant insights into how influxes of sodium, calcium, and potassium ions mediate secondary axonal damaged after a SCI just working *in vitro* [165, 166]. Moreover, Bradbury *et al.* demonstrated that chondroitinase ABC is able to restore post-synaptic activity below the lesion site after electrical stimulation of corticospinal neurons *in vivo*

[161]. Usually, electrophysiology testing is performed in anesthetized animals; however, it is also possible to execute neurophysiologic recordings while animals perform other behavioral tests (known as contact electrode recording) [167]. The main disadvantages of electrophysiology are the need of specialized devices and operators, its invasiveness, and the fact that the testing cannot be repeated daily or weekly (except for the contact electrode recording test). Nevertheless, these electrophysiologic techniques will most likely continue to play a role in the assessment of therapeutic approaches, especially in conjunction with other outcome tests.

9. Novel Strategies for SCI Repair

As described in section 4 of the present chapter a combination of factors is responsible for the lack of neural regeneration and minimal functional recovery usually observed after SCI. Given the multifaceted nature of SCI, many conceptually different paths to facilitate recovery have been investigated. In this section we discuss the diverse therapeutic approaches that are being tested for SCI.

9.1. *Cell Therapy*

Motivated by the physical consequences of the World War II, the era of cell transplantation began with the landmark work of Georges Mathé, which transplanted bone marrow to six persons exposed to high doses of radiation [168]. More than sixty years later cells derived from the bone marrow are still the ones frequently used in clinical transplantation. Nevertheless, cell transplantation approaches have, since then,

been extensively investigated for a wide range of diseases/injuries. In the particular case of SCI, cells such as neural stem cells (NSCs), mesenchymal stem cells (MSCs), embryonic stem (ES) cells, olfactory ensheathing cells (OECs), Schwann cells (SCs) and more recently, induced pluripotent stem (iPS) cells, already provided valuable pre-clinical data about their regenerative potential in SCI animal models. The properties, advantages and disadvantages of each of these cells are further explored below.

9.1.1. Neural Stem Cells

Neural stem cells are multipotent cells with the ability to differentiate into neurons, oligodendrocytes, and astrocytes. These cells are present in the adult and developing CNS and can be isolated and expanded *in vitro* (self-renewing cells that are often cultured as neurospheres) [169]. The rationale behind the use of NSCs in SCI treatments focus on the replacement of lost tissue (mainly neurons and oligodendrocytes) by these cells, as well as by the provision of trophic support to the survival neuronal tissue. Indeed, it was demonstrated that NSCs are able to secrete several neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF), *in vitro* and *in vivo* [170]. However, when NSCs were transplanted to normal or injured adult rat spinal cord either remained undifferentiated or differentiated along the glial lineage [171, 172]. Neuronal differentiation is rarely observed in the spinal cord. The Gage lab demonstrated that *in vivo* differentiation of NSCs is extremely related with environments cues. They showed that adult spinal cord-derived NSCs implanted into a neurogenic region (granular layer of the hippocampus) differentiated in neurons; however, the same kind of cells transplanted into the adult

spinal cord only give rise to glial cells [172]. Remaining undifferentiated or differentiated predominantly into glial cells is a potential problem for the direct transplantation of NSCs into the injured spinal cord. Nevertheless, functional improvements assessed by the BBB locomotor scale have been reported by several authors after the transplantation of NSCs in SCI animals [173-176]. Moreover, an interesting approach proposed by Fisher lab is the use of a mix population of neuronal-restricted precursors (NRPs) and glial-restricted precursors (GRPs). Restricted precursors cells are different from NSCs since they are committed to a specific lineage (give rise just to neurons or just to glial cells). The mixed population can be obtained either by directed isolation from fetal spinal cord or by pre-differentiation of NSCs *in vitro*. This experimental design improved cell survival and migration, increased cell differentiation into neural and glial phenotypes and ultimately led to motor improvements of SCI animals [177, 178].

A plethora of different origins NSCs has been described, either isolated from embryonic or adult spinal cord and brain regions. Generally, these cells integrated well in the host spinal cord and usually led to an improved motor behavior. The translational studies to humans recently start with the recruiting of SCI patients for a phase I/II clinical trial to be held in Switzerland and sponsored by the biotechnology firm StemCells Inc. (source: clinicaltrials.gov; clinical trial identifier: NCT 01321333). Nevertheless, the absence of a full understanding about the mechanisms through which NSCs provide functional benefit conjugated with practical issues hindering their isolation and differentiation as well as some ethical concerns (namely when fetal NSCs are used) greatly contribute to the absence of more clinical trials using NSCs in SCI patients. A much more detailed characterization, especially of the adult NSCs and their application in different injury

models, severities, and treatment intervals will most likely increase the translation studies of these cells.

9.1.2. *Mesenchymal Stem Cells*

Adult tissues, such as bone marrow, adipose, umbilical cord and others represent plentiful, ethical, and easily accessible sources of MSCs for regenerative applications. These multipotent cells are particularly appealing for SCI repair because they are easy to isolate and expand without serious ethical and technical problems. Moreover, the MSCs are known to present low immunogenicity, have an anti-inflammatory/immunosuppressant effect, do not form tumors, have a pathotropic action, and by the ability to be transplanted in an autologous way [179]. The MSCs derived from the bone marrow (bmMSCs) are perhaps the most widely applied stem cells in SCI experiments. Several laboratories qualify these cells as pluripotent since they demonstrated that bmMSCs had the ability to differentiate into neurons and glia cells [180-182]. However, these findings have not been confirmed and some authors argue that cell fusion and transdifferentiation is happening rather than cell differentiation [183-185]. Approximately 50% of the experimental studies employing bmMSCs in SCI animal models led to some motor improvements [186]. In the majority of the studies the cells were injected into or next to the spinal cord lesion site, however, some authors demonstrated that bmMSCs can be successful delivery intrathecally [187] and even intravenously [188]. In a work performed by Deng and colleagues an impressive BBB score of 13 is attributed to SCI rats injected with human bmMSCs versus a score of 6 in control animals [189]. In an interesting study, Zurita and Vaquero transplanted autologous

bmMSCs 3 months after the injury and evaluated the animals for up to 12 months. They reported an improvement to a BBB of around 17, whereas the control animals were completely paralyzed throughout the time of the experiment (BBB=0) [190]. Such result of the control animals is very unusual since typically controls score a few points in the BBB even in short time experiments. Pre-clinical experiments in large SCI animals such as pigs and nonhuman primates have been carried out and animals transplanted with bmMSCs improved their locomotor performance. The potential mechanism by which bmMSCs may be acting it remains fairly known; however neurotrophic factor and anti-inflammatory cytokines secretion have been proposed [188, 191, 192].

From a translational perspective, clinical trials that reported the transplantation of bmMSCs into SCI patients did not reveal any major motor improvement up to one year after the surgery [193-195]. Unfortunately, these reports included small patient cohorts and were mostly not controlled. Thus, a systematic clinical validation is needed. Nevertheless, these first trials were extremely important in establishing the safety and feasibility of the clinical use of bmMSCs given that no serious complications were reported. Moreover, these translational studies have continued, for instance, at least two more clinical centers are recruiting SCI patients for this purpose (NCT01446640 and NCT 01328860) and another two clinical trials are currently active (NCT 01162915 and NCT 01325103).

Another possible source of MSCs is the umbilical cord and the adipose tissue. These cells share many similarities with the bmMSCs and therefore their potential for SCI regeneration should be more explored. Umbilical cord derived cells are usually obtained from a gelatinous matrix enclosed by the epithelial cells known as Wharton's jelly (WJ-

MSCs) [196]. Some authors demonstrated that WJ-MSCs are more highly proliferative and thus can be more rapidly and extensively propagated than adult bmMSCs [197, 198]. Moreover, Yang *et al.* demonstrated that the transplantation of human WJ-MSCs in full transected rats led to axonal regeneration across the injured area as well as to an increase of trophic factors. These findings were associated with functional motor improvements [199].

With respect to MSCs derived from the adipose tissue (ASCs), which can be easily obtained from subcutaneous areas through a lipoaspiration surgery, some authors also claim that these cells have a higher growth rate when compared to bmMSC [200]. Regarding the *in vivo* experiments, the transplantation of these cells in a contusion SCI rat model revealed to promote functional recovery both in the BBB and grid test and decrease cavitation of spinal cord tissue [201]. Surprisingly, and in spite of the limited understanding of efficacy, safety and feasibility of ASCs, one clinical trial was already conclude in Korea using this cells in SCI patients (NCT01274975). No data is available until now, however, clearly both adipose and umbilical cord derived MSCs needed much more pre-clinical data in order to be considered for clinical trials.

9.1.3. *Olfactory Ensheathing Cells*

Olfactory ensheathing cells are a glial cell type which plays an important role in the lifelong neural regeneration capacity of olfactory neurons. They support the constant regeneration of olfactory axons from the PNS (the olfactory mucosa) into the CNS (the olfactory bulb) [202]. In this sense, OECs hold great promising for SCI treatment since, it is possible that they can create a permissive microenvironment for axonal regeneration

across the lesion site. OECs can be obtained through nasal biopsies and, either implanted right way in conjugation with the cell matrix and/or other cells, or dissociated and cultured until sufficient cell numbers are obtained [203, 204].

After the implantation of OECs into a complete thoracic transection injury model, Ramon-Cueto and co-workers were able to demonstrate regeneration of corticospinal axons and improvements in animal motor behavior [205, 206]. Moreover, Raisman and Li demonstrated both in discrete and chronic SCI models that OECs were able to promote corticospinal regeneration and functional recovery [207, 208]. These studies gain considerable international attention and many others followed them. Improved function using xeno or allotransplanted OECs has been observed after incomplete and complete lesions, in chronic and acute SCI animals [209-213]. However, it is important to note that there are a significant number of experiments that fail to demonstrate any therapeutic action of OECs after transplantation in SCI models [214-217]. Yet, about two thirds of the experimental studies reported improvements in behavior outcome [186].

The mechanisms underlying the improvements observed after OECs transplantation are still in debate. It was demonstrated that, after implantation, the OECs are able to migrate in the white and gray matter as well as through the glial scar [206, 218]. However, it is yet to be demonstrated if the OECs migration trails the axon regeneration preventing in this way the axons to respond to the inhibitory signals found in the injury site. Furthermore, Takami and colleagues demonstrated that the OECs led to a decrease in the astrocytic response and a decrease on the expression of the growth-inhibitory molecules CSPGs [217]. Others also demonstrated that OECs are able to secrete neurotrophic factors such as NGF and BDNF [219]. A controversial subject is related with the ability

or not of the OECs to remyelinate injured axons. Previous experiments showed enhanced myelination after OECs transplantation [220, 221], however, the possibility that the myelin formed by derived Schwann cells rather than OECs remains to be excluded [222]. The preclinical data in conjugation with the possibility of autologous transplantations make these cells attractive for translational studies. Indeed, it was already reported that more than 400 SCI patients were transplanted with OECs [203, 204, 223, 224]. Feron *et al.* reported the absence of complication following 3 years after transplantation; however, no improvements were detected [203]. Furthermore, Lima and colleagues, in an unblinded, non-randomized trial, reported improvements in 11 of the 20 chronic SCI patients being the condition of 1 patient deteriorate after the trial [204]. It must not be overlooked that this intervention was combined with a strong rehabilitation regimen and no controls patients were used. Additionally to these trials, physicians from Poland are now recruiting SCI patients for another clinical trial (NCT 01231893). These studies are important to help us disclosing about OECs safety and feasibility. However, it seems that more preclinical data is needed, especially in contusion/compression SCI models, in order to establish whether there are conditions under which transplantation of OECs promote plasticity, neuroprotection, regeneration and/or functional recovery.

9.1.4. Schwann Cells

Schwann cells (SCs) are known as the oligodendrocytes of the PNS, given that they produce the myelin sheaths that surround the PNS axons. Moreover, after an injury in the PNS, SCs play a key role in promoting axonal regeneration by three mechanisms: the secretion of a variety of growth factors, such as NGF, BDNF, NT3, CNTF, GDNF as

well as FGF; the expression on the membrane surface of adhesive molecules such as L1 and N-CAM, which are known to support axon growth; and the production of extracellular matrix molecules that also support axon growth, such as laminin, fibronectin and collagen [225-227]. It was the discovery of these *sui generis* characteristics generated interest in SCs as a potential candidate for SCI repair.

Of all cells types being used in SCI therapies, SCs have the longest history of transplantation, being the first experiment proposed by Bunge and colleagues in 1981 [228]. Since then, the efficacy of Schwann cells in promoting axonal regeneration and myelination has been extensively studied in a variety of experimental models [226, 229]. For instance, Takami *et al.* demonstrated that after implantation of SCs into SCI animals the cavitation is reduced and sensory and spinal axons extend into grafts, and many are myelinated. Moreover, functional recovery was also reported [217]. Others demonstrated that implanted SCs cause extensive infiltration of endogenous SCs to the site of injury [230]. In fact, it was recognized that cell transplants, not just SCs but also skin-derived precursors and blockers of inhibitory molecules facilitate the invasion of host SCs into the injured spinal cord [151, 231]. This invasion of endogenous cells suggests that host SCs may contribute to the recovery observed in other therapies.

Surprisingly, it has been shown that when SCs were used alone their migration into the CNS is stopped after contact with astrocytes [232]. Additionally, corticospinal tracts show a delayed and poor regeneration activity after SCs transplantation [233], and finally, SCs transplants do not allow axons that enter into grafts to exit and reenter on the host spinal cord [164]. As a result of these limitations, SCs have been used in combination with other co-treatments such as neuroprotective agents, blockers of

inhibitory molecules or neurotrophic factors [234-236]. These combination experiments led to superior therapeutic outcomes, which suggest that SCs “need a friend” that minimize the inhibitory nature of the lesion site (please see the combinatory therapeutic approaches section - 9.3.1).

The translational studies are not abundant using SCs. In fact it was only possible to found one clinical trial employing these cells and there are no signals that more will come (clinicaltrials.gov). In the study of Saberi and coworkers, autologous transplantation of SCs in just four SCI patients did not reveal any serious complications up to 1 year after the surgery. However, not functional outcome was reported [237]. In a clinical trial using multiple sclerosis patients, SCs revealed to be safe and feasible, as well as promoted remyelination [238]. It is clear that additional clinically relevant experiments are required to scrutinize the therapeutic potential of SCs in SCI.

9.1.5. *Embryonic Stem Cells*

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst. These cells have the indefinitely ability of self-renew and can differentiate into cells from all three germ layers [239]. These characteristics make them an attractive cell source for SCI regeneration. However, ES cells are not directly transplanted in animal models, usually an *in vitro* pre-differentiation protocol is performed in order to obtain a desirable cell population [240]. McDonald and coworkers pre-differentiated mouse ES cells in neurons or oligodendrocytes and demonstrated both remyelination and functional recovery in contusion SCI animals [241, 242]. The Keirstead lab has focused on the pre-differentiation of human ES cells into oligodendrocytes progenitor cells (OPCs). The

implantation of OPCs in myelin-deficient *shiverer* mice demonstrated that these cells differentiated in mature oligodendrocytes and restored myelination [243]. Another study from the same lab but now using contusion injured animals demonstrated that OPCs transplantation lead to remyelination and locomotor functional recovery [244].

Nevertheless, there are several concerns regarding to the use of ES cells. First of all, ethical concerns were raised concerning to the destruction of human embryos in order to isolate ES cells. Furthermore, concerns about the safety of ES cells transplantation were raised due to the formation of teratomas [245]. Possible clinical applications of ES-derived cells critically depend on the ability of labs to produce pure differentiated cell populations *in vitro*. The U.S. biotechnology company Geron was until recently recruiting SCI patients to test the safety of embryonic-derived OPCs (NCT01217008). This clinical trial was originally approved by the FDA, then put on hold due to concerns of cyst formation, and then in October of 2010 approved again but not without considerable objection and controversy [246, 247]. In October 2011, Geron presented the first results of the ES derived cells clinical trial. The cells were well tolerated by the patients, with no serious adverse events. However, no neurological recovery was observed [248]. Despite of these results, that bring to light the possible safety of ES derived cells therapies, one month later the Geron administration surprisingly decided to shut down all the experiments involving ES cells claiming financial reasons [249]. Due to all the controversy around the use of ES cells, researchers tend to choose other sources of stem cells for regenerative therapies.

9.1.6. *Induced Pluripotent Stem Cells*

In 2006, Takahashi and Yamanaka demonstrated that pluripotent cells could be obtained from fibroblast by the introduction of four genes into cells via retrovirus-mediated gene transfer [250]. Subsequently, others demonstrated that induction to pluripotency can also be performed without the need of potential hazardous viral vectors through the use of chemical and small molecules [251], proteins [252], non-integrating excisable virus [253], and drug selectable targeting [254]. The resulting cells, termed induced pluripotent stem (iPS) cells demonstrated to have many of the characteristics of ES cells, such as such as tri-lineage differentiation and generation of viable chimaeras [250]. However, the use of iPS cells owns several advantages over the ES cells. They circumvent the ethical concerns associated with ES cells and allow autologous transplantations of pluripotent cells. Nevertheless, iPS and ES cells also share similar disadvantages such as the ability to form teratomas [250, 255].

An increasing number of experiments are been conduct with iPS cells in SCI models. Recently, two experiments from Okano lab demonstrated that NSCs derived from iPS cells implanted in immunodeficient contusion mice result in trilineage neural differentiation, functional recovery and no tumor formation [256, 257]. Much more studies are required to confirm the safety and feasibility of iPS cells before clinical a can be initiated.

9.2 Molecular Therapy

The remarkable advances in molecular biology in the past decades have led to a better understanding of the mechanisms implicated in the pathophysiology of SCI as well as mechanisms that control axonal growth and sprouting. This knowledge has raised the hope of developing new therapies that can attenuate secondary damage and maximize regeneration after a SCI. Molecular therapies usually focus on the modulation of inflammatory response, administration of growth-stimulating factors and/or blocking the inhibitory nature of the injured adult CNS tissue. In this section approaches that, by lowering the extrinsic inhibitory cues or increasing intrinsic growth promoting cues, accomplished various degrees of anatomical growth often correlated with improved behavioral outcomes, will be reviewed.

9.2.1. *Protecting the Spinal Cord*

As mention above (see section 4 of this chapter) the pathophysiology of a SCI is very severe and complex, playing a crucial role on the failure of axonal regeneration. While the use of the anti-inflammatory drug methylprednisolone is very controversial with no robust evidences of clinical benefits and several side effects, researches are now focusing on how to exploit the constructive effects of inflammation and at same time avoid the destructive effects.

In this context, Bethea and colleagues proposed the use of **interleukin-10** (IL-10), an anti-inflammatory cytokine capable of inhibiting the inflammatory reactions of monocytes and macrophages in the PNS, and capable of inhibiting the production of

TNF- α by astrocytes in CNS, in a SCI condition [258]. They demonstrated that the systemic administration of IL-10 significantly reduces the lesion volume by approximately 50% and this was correlated with enhanced functional recovery [258]. More recently experiments also demonstrated that IL-10 promotes direct neuronal survival, provide trophic support to neurons and limits the onset and progression of injury-induced pain behavior [259-261]. It is important to note that, in one study conducted by Takami and colleagues the administration of IL-10 led, indeed, to reduction a of gray matter damage but did not improve functional outcomes [262]. In this sense, further pre-clinical experiments are needed before the beginning of clinical trials using IL-10 in SCI patients.

In the last decade, the broad-spectrum antibiotic **minocycline** has sparked significant interest in the neuroscience community for its potential role in pos-traumatic neuroinflammation. The ability of minocycline to inhibit microglial activation, inhibit the release of pro-inflammatory mediators and to have an anti-apoptotic action has made it an attractive agent to study in SCI models [263, 264]. Wells and colleagues in 2003 were the firsts describing the beneficial effects of minocycline in SCI rodents. Minocycline treatment increased rubrospinal tracts, reduce lesion area and improve functional outcome in BBB scale and inclined plane test [112]. After this initial study, it was demonstrated in different labs that minocycline treatment reduces cavitation, apoptotic cells, caspase-3 activity, cytochrome c release, necrosis and gliosis [265-267]. Moreover, the neuroanatomic improvements were correlated with functional beneficial outcomes. However, more recently, three studies failed to demonstrated beneficial effects of

minocycline in SCI models [268-270]. One study, in particular, was a NIH sponsored formal replication attempt that was unable to reproduce previous results [268]. Despite of this inconsistency a human clinical trial was initiated in 2007 in Calgary, Alberta (Identifier: NCT005594494). No published analysis of data is available; however, a large scale multicenter randomized study sponsored by Rick Hansen Institute is already planned in Canada [271].

Due to the known side effects of steroids agents, several non-steroidal anti-inflammatory drugs (**NSAIDs**) are been tested in SCI models. The most widely used are **indomethacin** and **ibuprofen**. Many of the indomethacin studies showed decreased edema, less tissue damage and less microglia and astrocytes [272-274]. The majority of the studies do not assessed behavioral outcomes, and the results of the few that did are contradictory [275, 276]. Additionally, the study of Guven and colleagues also show that indomethacin significantly increased lipid peroxidation, suggesting a harmful role for this drug [277]. Ibuprofen, however, has more recently been found to promote histological and behavioral improvements even when administrated 3 and 7 days post injury [278, 279]. Moreover, ibuprofen also reduced RhoA activation, which may indicate that this drug, in conjugation with attenuation of inflammation, can promote axonal sprouting [279]. A controversial issue of these pre-clinical experiments is the dose used. The conversion of the therapeutic dose used in rats to a human equivalent dose suggests that it was used a concentration 5 to 7 times higher than what is known to be clinically safe [280].

Recently, pre-clinical experiments using the FDA approved drug **atorvastatin** (Lipidor) revealed beneficial effects when administrated in SCI models. Atorvastatin is commonly used for the treatment of high cholesterol, triglyceride levels and prevention of heart attacks and stroke. It was found by Pannu and colleagues that atorvastatin reduces the expression of inflammatory cytokines, macrophage invasion, GFAP reactive astrocytes and apoptotic cells. Moreover, atorvastatin treatment also promoted tissue sparing and significant locomotor recovery [281, 282]. Déry *et al.* also demonstrated that atorvastatin prevented the elevation of caspase-3 activity and significantly improved locomotor outcome [283]. However, more pre-clinical experiments should be performed for this drug before any clinical trial, since, a recent attempt to replicate these results failed to demonstrated any beneficial outcome [284].

The hematopoietic growth factor **erythropoietin (EPO)** is produced in the kidney and stimulates proliferation and differentiation of erythroid precursor cells [285]. However, it is also known to exert non-hematopoietic effects in the CNS. For instance, EPO has a trophic effect on cholinergic neurons, influencing their differentiation, maintenance and regeneration [286]. After SCI, EPO administration has shown a neuroprotective effect, namely, by reducing apoptosis, inflammation, lipid peroxidation and by increasing white and gray matter sparing [287-289]. Many authors also reported improved locomotion scores and improved function on the swimming test. However, these beneficial effects were not always found [290, 291]. A NIH-sponsored attempt to reproduce the positive effects of EPO failed to demonstrate both histological and behavioral outcomes [291]. Nevertheless, a modified version of EPO (lacking hematopoietic effects) was being test

in SCI patients, although, the Italian medicine agency recently suspended the trial without public explanations (NCT00561067).

It has been long understood that neuronal damage is mediated by disproportionate intracellular accumulation of sodium and calcium ions. This accumulation is mediated by overstimulation of NMDA receptors (due to high glutamate levels after injury) in a process known as “excitotoxicity” [165, 292]. In order to protect neural tissue several researchers explored the therapeutic potential of either sodium channel blockers, such as **riluzole**, or NMDA antagonists, such as **magnesium**.

Riluzole is a FDA-approved drug for the treatment of amyotrophic lateral sclerosis that has been found to promote tissue sparing, decrease lipid peroxidation, reduce neuronal loss, enhance electrophysiologic recordings and improve functional recovery [124, 293-295]. Of note, in a study by Kitzman and colleagues, higher dose of riluzole was associated with undesired side effects, such as lethargy and locomotor ataxia [296]. Encouraged by preclinical results and facilitated by the fact that oral riluzole is FDA-approved; a multicenter clinical trial for acute human SCI is ongoing in North American (NCT 00876889).

Magnesium is a natural mineral that play an important role in several functions of the human body. It was demonstrated that magnesium levels decrease significantly within the injured spinal cord [297], and that the severity of injury was closely correlated with magnesium levels in plasma and cerebrospinal fluid [298]. Moreover, the fact that magnesium is able to block NMDA receptors encouraged its use in SCI models. Results revealed that magnesium can increase tissue sparing, reduce apoptosis, lipid peroxidation,

restore BBB integrity and improve functional outcome [299, 300]. It should be noted that the doses used so far exceed the tolerable by humans. In this sense, formulations of magnesium within polyethylene glycol (PEG) were been developed and revealed that PEG allows that a much lower dose of magnesium can be applied effectively [301]. This PEG formulation is commercialized by Medtronic and will be used in a Phase II multicenter clinical trial.

In the fall of 2007, Kevin Everett, a NFL football player, suffered a cervical SCI and achieved significant neurologic recovery after **systemic hypothermia** treatment [302]. This well-publicized case triggered the resurgence of interest in a treatment that has fascinated scientist for many decades [303]. While technically not a “molecular therapy”, systemic hypothermia was included in this review due its effects on neuroinflammation and neuroprotection. Hypothermia provides protection to neural tissue by slowing basic enzymatic activity, thus reducing the energy requirements of the cells and maintaining intracellular ATP concentrations [304, 305]. Experiments in SCI models demonstrated that moderate hypothermia treatment (30-34°C) attenuate neutrophil invasion, reduce oxidative stress, apoptosis, vasogenic edema, and tissue damage [306-309]. Although the majority of the studies do not report any behavioral outcome, functional improvements have been described by some authors [306, 308]. As far as we know, the only side effect of hypothermia treatment was published by Strain and colleagues, which observed that hypothermia significantly increased gastric ulcerogenesis [310]. The first clinical trial using hypothermia in human SCI (14 patients) was conducted by investigators from the Miami Project, which verified the safety of this treatment, seeing that, hypothermia do

not increased the incidence of secondary complications usually observed in these patients. Moreover, they observed that, although without statistical significance, 42.8% of the patients recovery from a complete stage to incomplete SCI stage [311, 312]. Due to these promising findings, a large-scale trial of systemic hypothermia is now being organized.

9.2.2. *Overcoming the inhibition*

As discussed above (see section 4 of this chapter) the pathophysiology of SCI goes well beyond of inflammation, edema and cell death. The axonal regeneration which would be necessary for re-establishing connectivity across the injury site is inhibited by a number of features within the injured CNS. Myelin, for instance, contains several inhibitory molecules that hold back axonal growth. Additionally, within days after injury, a glial scar is formed around the injury site, producing a physical barrier to axonal regeneration. In order to overcome these inhibitors to axonal growth a number of therapeutic strategies have been proposed.

In 1988 Schwab and Caroni reported the first evidence of the presence of molecules in the myelin that inhibit axon growth [313]. Among them, one of the most potent inhibitor was a 250-kDa glycoprotein than was later named **Nogo** [314]. Subsequently, they generated and administrated in SCI models a blocking **anti-Nogo antibody**, which was associated with improved behavioral outcomes on a variety of tests, such as open field locomotion, rope climbing and food pellet reaching. Moreover, anti-Nogo treatment led to substantial axonal sprouting and long-distance corticospinal regeneration [315-317]. Nevertheless, a study from Oudega and colleagues demonstrated that blocking Nogo does

not promote the regeneration of sensory axons suggesting that future treatments will have to include the neutralization of other inhibitory molecules [318]. Moreover, an independent study sponsored by the NIH failed to replicate the beneficial outcomes observed after the administration of NEP1-40 (an anti-Nogo peptide generated in Strittmatter lab) [319], suggesting that the use of antibodies may be more valuable in this situation. The anti-Nogo antibody from the Schwab group, on the other hand, demonstrated to promote corticospinal tract axons regeneration also in marmoset monkeys [320]. The pre-clinical data generated by Schwab lab were undergoing translational human testing in a clinical trial sponsored by Novartis (Identifier: NCT00406016). At the time of writing, results of this study have yet to be published.

After the discovery of the Nogo glycoprotein other myelin-associated inhibitory molecules were identified in CNS. Among them were the myelin-associated glycoprotein (**MAG**) and the oligodendrocyte myelin glycoprotein (**OMgp**) [321]. Surprisingly, it was demonstrated that both MAG and OMgp binds with high-affinity to the Nogo receptor (NgR) [45, 322], suggesting that the different inhibitory molecules within the myelin activated the same intracellular signaling pathway. Then, unlike the anti-Nogo antibody, which is specific to the Nogo protein, a single pharmacological intervention targeting this intracellular pathway might neutralize the effect of many myelin inhibitors.

It was demonstrated that the point of convergence for a single therapeutic intervention might be on the **Rho** pathway [323]. Rho is a small guanosine triphosphatase (GTPase) that plays an important role in transducing extracellular signals to changes in cytoskeletal proteins [324]. Therefore, the activation of Rho leads to the depolymerization of actin

filaments and subsequently growth cone collapse. On the other hand, the inhibition of Rho leads to the polymerization of actin making possible the axonal growth [323].

The landmark work of Jalink and colleagues demonstrated that inhibition of Rho pathways using **C3 transferase** promotes neurite outgrowth [325]. Moreover, administration of C3 transferase also demonstrated to have a beneficial effect *in vivo*. Dergham and colleagues verified that animals treated with C3 presented long-distance regeneration of corticospinal axons, which was correlated with beneficial functional outcomes [326]. These results were confirmed by others labs [327] but not in all studies [328]. Nevertheless, experiments found in literature suggest that either the direct inhibition of Rho or selective Rho kinase or even non-selective protein kinase have a beneficial effect in axonal regeneration and locomotor behavior [328-330].

On the basis of these preclinical findings, a multicenter clinical trial, headed by Lisa McKerracher and sponsored by BioAxone Therapeutics Inc. (NCT00500812) initiated in 2005, demonstrated that **Cethrin** (commercial name for a Rho antagonist) slightly improved the motor recovery without serious adverse events [331]. Such results have encouraged the planning of a subsequent large randomized clinical trial (NCT00610337) that was prematurely terminated because of insufficient funds.

An important class of inhibitory ECM molecules is the chondroitin sulfate proteoglycan (**CSPG**). Activation of astrocytes in injured spinal cord is associated with changes in the ECM, being the CSPG an important component of this matrix and having a substantial inhibitory effect on the endogenous regeneration [332]. A number of pre-clinical experiments, mainly from Fawcett lab, demonstrated that degradation of CSPGs with the

chondroitinase ABC (**ChABC**) enzyme can render a more permissive environment for axon regeneration (both ascending sensory projection and descending motor projection), can restore post-synaptic activity below the injured site and can promote functional recovery of locomotor and proprioceptive behavior [161, 333, 334]. More recently, the work of Garcia-Alias and colleagues evidenced that ChABC treatment opens a window during which rehabilitative training supports functional improvements [335]. However, significant improvements with ChABC alone were not observed by others [336]. Some authors demonstrated, yet, that ChABC may play an important role as an adjunct therapy for cell transplants [336, 337]. The ChABC translational studies in SCI humans did not start yet. The Seikagaku Corporation decided not to publish the results of the safety and efficacy tests of ChABC in non-human primate models of SCI. The Japanese Corporation announced in November 2011 that due to the promising results of a phase II clinical trial using ChABC for the treatment of lumbar disc herniation, a phase III clinical trial will be initiated [338]. This seems to indicate that ChABC is safe for humans; nevertheless, tests in SCI patients are still required.

There are other molecules that also display an inhibitory effect after SCI. However the amount of preclinical experiments employing these molecules is still reduced, and so, they will not be deeply discussed here. Nonetheless, many molecules from the **semaphorins** and **ephrins** family act as inhibitory or repulsive cues for axonal growth [339, 340]. The inhibition of such molecules in SCI models enhanced axonal regeneration, increased myelination (derived from higher invasion of peripheral Schwann cells), reduced apoptosis, and was correlated with better functional recovery [151, 341,

342]. Nevertheless, more preclinical data is needed to verify the robustness of these therapeutic approaches.

9.2.3. *Stimulating axonal growth*

Trophic factors are tightly linked to cell growth, guidance, and survival during the development. In CNS, **neurotrophic factors** are regulated both spatially and temporally, usually decreasing as the development proceeds. However, their expression persists throughout adulthood in many CNS regions associated with functional plasticity (hippocampus, cortex and olfactory bulb) [343]. Conversely, the spinal cord deals with a huge reduction in neurotrophins levels from embryonic state to adult maturation [343]. Nevertheless, the clear evidence that several growth factors play a central role on the axonal regeneration after a PNS injury made them an attractive therapeutic tool for SCI repair [344-346].

Neurotrophins have been capable to promote axonal regeneration and sprouting of both ascending and descending fibers but with two major constraints: deficient repair of corticospinal tracks and lack of regeneration through degenerative white matter [347]. Even so, **NGF** has demonstrated that is able to promote the sprouting and regeneration of cholinergic motor axons, primary nociceptive sensory axons (which may contribute to dysfunctional pain), and cerulospinal axons [348-350]. On the other hand, **BDNF** promotes regeneration of raphespinal, rubrospinal and reticulospinal motor axons and proprioceptive sensory axons [351-353]. **NT3** has an effect similar to BDNF, but might also have an effect on the corticospinal tract [354, 355]. These findings indicate that different populations of axons in the CNS show distinct patterns of growth factor

sensitivity, which is explained by the divergent profile expression of high-affinity tyrosine kinase receptors by different populations of neurons [356]. Other factors, such as **bFGF**, **VEGF**, and **GDNF**, also demonstrated to have a beneficial effect after administration in SCI models. However, more preclinical data using these factors are needed [357-359]. The majority of studies using this kind of treatment only reported on histological outcomes, and the studies in which behavioral outcomes were measured just described modest functional recovery. Moreover, the delivery of growth factors in chronic SCI models might be less effective due to the loss of appropriated receptors by injured neurons [360].

Unfortunately, a clinical trial using systemic delivery of NGF in diabetic neuropathic patients failed to demonstrated efficacy and safety [361]. However, in other clinical trial performed by Tuszynski and colleagues the implantation of autologous fibroblasts genetically modified to express NGF into the forebrain of Alzheimer disease patients do not result in adverse side effect and revealed to slightly reduced the rate of cognitive decline[362]. Furthermore, a clinical trial using bFGF in nine chronic SCI patients revealed to be safe and to promote sensory and motor recovery to some extent (difficult to distinguish from any possible placebo effect) [363].

Neurotrophins bind to the neuronal membranes via distinct tyrosine kinases receptors. However, the fact that neurotrophins signaling elevate the intrinsic levels of **cAMP** seems to indicate that distinct neurotrophins activated the same intracellular signaling pathway [364]. Then an intrinsic molecular approach targeting this pathway might result in higher beneficial effects than those observed with the use of a single neurotrophin. In

addition, exogenous elevation of cAMP has been shown to promote axonal regeneration when prophylactically applied [365, 366]. Obviously, this is not clinically relevant. Moreover, the administration of cAMP may result in undesirable side effects [367-369]. In order to avoid side effects, others strategies have been proposed to increase cAMP levels. For instance the use of **rolipram**, a molecule that prevents cAMP hydrolysis, has been show to attenuate oligodendrocytes death and to restore a respiratory pathway [370, 371]. The effect of rolipram has been more pronounced when in combination with cells [372, 373]; although this was not always the case [374]. These findings suggest that approaches aimed to increase the intrinsic levels of cAMP may be important in future treatments for SCI repair.

9.3. Combinatorial Therapies

9.3.1. Searching for synergistic effects

Due to the complexity of SCI there is the expectation among researchers that combinatorial therapies will be a more effective strategy to promote recovery than a single therapeutic approach alone. In this sense, therapies that combine cell transplantation, activation of growth promoting programs and/or attenuation of growth inhibitory pathways have been explored. After an extensive overview in literature, it was possible to find several studies using combinatorial therapies (see table 2), of which over half of them demonstrated synergistic effects between the subjects combined. For instance, Pearse and colleagues demonstrated that the transplantation of Schwann cells combined with the modulation of cAMP levels (both by direct injection of cAMP and

inhibition of cAMP hydrolysis by injecting rolipram) enhances axonal sparing and myelination, promotes growth of serotonergic fibers into and beyond grafts, and significantly improves locomotion [372]. Moreover, the combination of autologous PNS bridges and ChABC showed to work synergistically in three different studies. Using this strategy it was possible to accomplish superior axonal regeneration and extension for longer distances, functional synapses, significant motor improvements, and restoration of the respiratory pathways in a level not possible to achieved using a single therapy alone [375-377].

The use of cells in combination with other therapeutic approaches is the most commonly found in literature, being the SCs, bmMSCs and OECs the most frequently exploited. Normally, these cells are used in combination with trophic factors, being the NT3, BDNF and FGF often applied. Unfortunately, approximately one third of the studies using combinatorial approaches did not assesse for synergistic effects between the components employed. In these studies the authors usually demonstrated that the combined treatment lead to a better outcome than using one component alone, however, they did not controlled the experiment for all possible combinations of components. In this sense, it is impossible to conclude about promising synergistic effects.

Few studies published negative data, however, the combined use of OECs and SCs failed to demonstrate any synergistic effects in two different experiments [217]. Furthermore, this combination seems to lead to some antagonistic effects on the role of SCs, since only the SCs alone were able to promote functional recovery. However, Fouad and colleagues showed that this negative result may be overcome by matrix modification. The combinatorial treatment employing OECs, SCs, and ChABC significantly improved

motor function and increased the number of myelinated axons and serotonergic fibers that grew through the graft and into the caudal spinal cord [337].

Bretzner and colleagues also demonstrated that OECs in combination with cAMP did not provide additional benefits compared with either treatment alone. On the other hand, the use of OECs and rolipram resulted in greater axonal density within the graft and improved motor performance in a cylinder test [373].

The combinatorial therapy is a very promising approach to treat the injured spinal cord; however, more preclinical data is needed to fully understand the beneficial or detrimental effects of the therapies that are being proposed. Nevertheless, one clinical trial using autologous bone marrow mesenchymal stem cells combined with granulocyte macrophage-colony stimulating factor (GM-CSF) was already performed in South Korea and showed some promising results [378]. No serious clinical events were observed and 30.4% of the acute and subacute patients improved from ASIA A to B or C. The patients with chronic injuries did not improve. Long-term and a large scale multicenter clinical study is required to determine the precise therapeutic effect of this approach.

Table I.2. Combinatorial therapies aimed for SCI regeneration.

Reference	Methodology	SCI Model	Outcomes
Ziv, PNAS, 2006 [379]	NSCs + myelin vaccination.	Acute model of contusion.	Synergistic effects observed. Smaller lesion site in the CT group; Less activated macrophages and microglia; Significant motor improvements.
Karimi-Abdolrezaee, PNAS, 2006 [152]	NSCs + PDGF-AA + bFGF + EGF + Minocycline.	Chronic model of compression.	Synergistic effects observed but not assessed for all possible combinations. 51% of NSCs differentiated into oligodendrocytes and ensheathed the axons; Improved locomotion observed by BBB, grid-walk and footprint analyses.

Table I.2. Combinatorial therapies aimed for SCI regeneration. (continued)

Reference	Methodology	SCI Model	Outcomes
Meng, Cell. Biol. Int., 2008 [380]	NSCs + bFGF expressing AECs.	Subacute model of contusion.	Synergistic effects observed. Electrophysiological and locomotor improvements.
Ishii, J. Neurosci. Res., 2006. [381]	NSCs + anti-CNTF antibody	Acute model of hemisection.	Synergistic effects not assessed. Reduction of the glial scar; NSCs differentiation into neurons, oligodendrocytes and astrocytes; Increasing number of CST fibers; Behavioral function not assessed.
Kim, Acta Neurochir., 2006. [382]	bmMSCs + bFGF.	Subacute model of contusion.	Synergistic effects not assessed. Cavity volume decreased in CT group; CT promotes significant motor improvement.
Lu, Exp Neurol., 2005. [383]	bmMSCs + BDNF.	Acute model of transection.	Synergistic effects not assessed. CT enhanced the growth of serotonergic, coeruleospinal and sensory axons. However, functional recovery was not observed.
Lu, J. Neurosci., 2005. [384]	bmMSCs + NT3 + cAMP	Acute model of transection.	Synergistic effects observed. Sensory axons regenerated into and beyond the lesions in CT group only. Functional outcomes were not observed.
Chiba, Neuropathology, [385]. 2010	bmMSCs + ROCK		Synergistic effects observed in CST regeneration only. ROCK did not increase the therapeutic effects of bmMSCs on motor activity.
Fang, PLoS One, 2010. [386]	bmMSCs + PACAP	Subacute model of contusion.	Synergistic effects observed. CT increased the number of neuronal fibers, and increased the levels of antioxidant enzymes in the injury site, leading to better functional recovery.
Yoon, Stem Cells, 2007. [378]	bmMSCs + GM-CSF.	ASIA A patients.	Synergistic effects not assessed. No serious clinical events; 30.4% of the acute and subacute patients improved from ASIA A to B or C; No significant improvements in chronic group.
Deng, Cytotherapy, 2008. [189]	OECs + bmMSCs.	Acute model of contusion	Synergistic effects observed. Significant motor improvements observed by electrophysiology and BBB test.

Table I.2. Combinatorial therapies aimed for SCI regeneration. (continued)

Reference	Methodology	SCI Model	Outcomes
Ruitenber, Brain, 2005. [387]	OECs + NT3.	Acute model of hemisection.	OECs alone or in combination with NT3 decreased the lesion volume. Anterograde tracing showed a significantly greater number of distal CST axons only in the CT group. Treatment do not improved functional outcomes.
Bretzner, J. Neurosci., Res., 2010. [373]	OECs + cAMP or rolipram	Acute model of compression.	CT of OECs plus rolipram resulted in greater axonal density within the graft and improved motor performance in a cylinder test. OECs plus cAMP do not resulted in synergistic outcomes.
Takami, PNAS, 2002 and Pearse, Glia, 2007 [217, 388]	OECs + SCs	Subacute model of contusion	Both studies fail to demonstrated synergistic effects between OEC and SCs. Myelinated axons were found within regions of SCs but not OECs; More retrograde traced axons in SCs and SCs/OECs group than in OECs alone; Significant motor improvements only observed in SCs group.
Cao, Brain, 2004. [389]	OECs + SCs.	Acute model of transection.	Synergistic effects not assessed. Increased amount of fibers in the lesion site. CST regeneration. Functional recovery observed in the BBB and inclined plane tests.
Fouad, J. Neurosci., 2005. [337]	OECs + SC + ChABC.	Acute model of transection.	Synergistic effects not assessed. Increased number of myelinated and serotonergic fibers in the CT group. Significant improvements observed in BBB and in forelimb/hindlimb coupling test.
Menei, EJN, 1998. [390]	SC + BDNF	Acute model of transection.	Synergistic effects not assessed. More axons in the CT grafts than SCs alone. More retrogradelly labeled neurons in the CT group. Functional outcomes not assessed.
Pearse, Nat. Med., 2004. [372]	SC + cAMP + rolipram.	Subacute model of contusion.	Synergistic effects observed. CT promotes significant supraspinal and proprioceptive axon sparing and myelination. Growth of serotonergic fibers into and beyond SCs grafts. Significant improvements in locomotion observed by BBB, gridwalk testing and footprint analysis.

Table I.2. Combinatorial therapies aimed for SCI regeneration. (continued)

Reference	Methodology	SCI Model	Outcomes
Xu, Exp. Neurol., 1995. [234]	SC + BDNF + NT3	Acute model of transection.	Synergistic effects observed. Significantly more myelinated fibers in the CT group. More retrogradely labeled neurons in the CT group. Functional outcomes not assessed.
Guest, J. Neurosci. Res., 1997. [391]	SC + IN-1 or aFGF	Acute model of transection.	Synergistic effects not assessed. SC grafts alone do not supported CST regeneration and do not prevented die-back. SCs plus IN-1 supported some sprouting but die-back continued. SCs plus aFGF supported regeneration and reduce die-back. Functional outcomes not assessed.
Nikulina, PNAS, 2004. [392]	Fetal tissue + rolipram	Acute model of hemisection.	Synergistic effects not assessed. CT resulted in an increase axon regrowth into the transplant and reduces gliosis. Significant improvement in motor function was observed.
Kimura, Neurol. Res., 2005. [393]	ES cells + thyroxine	Chronic model of contusion.	Synergistic effects not assessed. ES cells alone improved motor function. No additional improvement was observed with the use of thyroxine.
Cao, J. Neurosci., 2010. [394]	OPCs + CNTF	Subacute model of contusion.	Synergistic effects observed in histological and electrophysiology outcomes, but not in motor behavior. CNTF significantly increased the percentage of adult OLs derived from grafted OPCs. CT increased the number of remyelinated axons and partially restored the conduction through the demyelinated fibers. Motor improvements observed using the BBB scale.
Houle, J. Neurosci., 2006. [377] Tom, J. Neurosci., 2009. [376]	PNS grafts + ChABC	Acute and chronic model of hemisection	Synergistic effects observed. More axons regenerated and extended for longer distance after CT than controls. Regenerating fibers were able to perform synaptics. Functional motor improvements observed after CT.
Alilain, Nature, 2011. [375]	PNS graft + ChABC	Acute model of hemisection	Synergistic effects observed. CT increased the regeneration of serotonergic axons. Recovery of diaphragmatic function and restoration respiratory pathways.

Table I.2. Combinatorial therapies aimed for SCI regeneration. (continued)

Reference	Methodology	SCI Model	Outcomes
Yick, J. Neurotrauma, 2004. [395]	ChABC + LiCL.	Acute model of hemisection.	Synergistic effects observed. CT significantly increased axonal regeneration and improved motor function in a greater extent than controls or single treatment.
García-Alías, J. Neurosci., 2011. [396]	ChABC + NT3 + NR2D expression	Acute model of hemisection.	Synergistic effects observed. More axonal sprouting in the CT group than single treatment alone. Electrophysiology improvements observed in the CT group only. CT animals presented more body stability and interlimb coordination than single treatment alone.
Bai, Eur. J. Physiol., 2010. [397]	ChABC + Clenbuterol	Acute model of transection	Synergistic effects observed. More axons myelinated by peripheral SCs in the CT group. CT promoted supraspinal axons regeneration and improved hindlimb function.
Nothias, Neurorehabil. Neural Repair, 2005. [398]	BDNF + NT3 + Serotonergic agonists + Exercise.	Subacute model of transection.	Synergistic effects observed. Individual treatments stimulated axonal growth and prevented muscle atrophy, but did not improved motor scores. Only CT resulted in improvements in motor function.
Klapka, EJN, 2005. [399]	cAMP + Iron chelator.	Acute model of hemisection.	Synergistic effects not assessed. CT improved CST repair and reduce axonal loss. CT resulted in functional recovery in the open field, horizontal ladder and in CatWalk test.
Ditor, J. Neurosci. Res., 2007.[400]	MgSO ₄ + PEG	Acute model of compression.	MgSO ₄ + PEG treatment increased dorsal myelin sparing, reduced lesion volume, improved locomotor recovery and reduced pain but did not provide additional benefit compared with either treatment alone.

9.3.2. *Tissue Engineering*

Tissue engineering (TE) holds great promising for the regeneration of different tissues, such as, bone, cartilage, nervous, muscular and others [401-406]. This interdisciplinary field combines the knowledge from physics, materials science, engineering, chemistry, biology, and medicine in an integrated manner with the ultimate goal of restore, maintain, or improve tissue function [407]. In order to do so, TE usually combines cells, bioactive molecules and biomaterials. The biomaterial, a 3D scaffold, plays a central role in any TE approach and should be carefully chosen.

For a successful result, scaffolds should possess a number of properties and characteristics defined as essentials in TE. Namely, scaffolds should: i) be biocompatible, i.e., it should not elicit an immune response after host implantation; ii) have desirable physical characteristics, this include, high porosity, large surface area, large pore size, fully interconnected geometry, and adequate mechanical strength; iii) be biodegradable, the ideal scaffold should only be totally degraded by the time the injury site is totally regenerated; and iv) have desirable surface properties, i.e., the scaffold should provide chemical and topographical signals to modulate cell organization. Taking in account all of these properties, scaffolds should be able to act as substrate for the initials phase of cell adhesion, guide cell behavior, such as migration, proliferation, differentiation, maintenance of phenotype and apoptosis, and, finally, temporarily act as a mechanical support for tissue regeneration [408-410].

Polymers, both from natural and synthetic origins, need to be processed in order to construct a 3D scaffold. Through the years a series of processing techniques were developed with the aim of producing scaffolds that can be used for SCI regeneration.

Among them are solvent casting techniques, phase inversion, fiber bonding, electrospinning, high pressure based methods, freeze drying and rapid prototyping technologies [411-414].

To date, 3D devices investigated for SCI repair include hydrogels, sponges, guidance tubes and nanofibrous scaffolds [415]. Hydrogels are viscous, crosslinked liquids that can be advantageous for filling the gap in a transected cord or filling the acellular cavity of contusion injuries. Hydrogels may also be used as vehicles to uniformly disperse living cells or drugs into the spinal cord [414, 416]. Moreover, some hydrogels have tissue-like mechanical abilities, similar to those of CNS tissue [417, 418], and can be easily modified to mimic the ECM matrix [419]. In contrast, sponges are highly porous 3D scaffolds with a wide surface area for cell transplantation [420]. Sponges have the disadvantage of mechanical weakness. Alternatively, tubes are porous hollow cylindrical channels that can guide regenerating axons and provide local, sustained release of therapeutic agents [421]. Finally, nanofibrous scaffolds, particular those with aligned fiber orientation, can provide a better environment for neural cell orientation, attachment, migration, proliferation, and differentiation than traditional scaffolds [422, 423]. However, the processing technique used to obtain these nanofibrous is limited to viscous polymer solutions and the scaffolds developed possess small pore size [424]. It is important to note that the use of scaffolds for SCI repair is still emerging, so, what constitutes an optimal scaffold remains to be defined.

Several authors have reported the improvement of both histologic and behavioral outcomes after the implantation of biomaterials (usually combined with cells or drugs) in

SCI models. For instance, Hejcl and colleagues reported that the implantation of the synthetic non-degradable hydrogel **PHPMA** (poly-(2-hydroxypropyl)-methacrylamine) modified with the amino acid sequence RGD and combined with bmMSCs in a chronic contusion SCI model improved both sensory and motor functions 6 months post-injury. Moreover, 5 weeks after the implantation the cavity was successfully bridged with axons myelinated by SCs and also with blood vessels and astrocytes [425]. Other three authors reported significant improvements in motor behavior after the implantation of guidance channels developed from the **PHEMA-MMA** (poly-(2-hydroxyethyl)-methacrylamine-co-methyl metacrylate) polymer [426-428]. In one study the scaffolds significantly promote the regeneration of sensory axons through the bridge [427], others reported a decrease in scar tissue formation [426]. However, other study described few invasion of regenerating axons into the channels [428].

Another successful approach using synthetic polymers is the implantation of the biomaterial **PEG** (polyethylene glycol). PEG is known to seal and repair cell membrane breaches produced by injury, decrease excitotoxicity, and inhibit the production of free radical [429-431]. The use of PEG micelles by Shi and colleagues showed to decrease lesion volume and attenuate macrophage response. These results were correlated with improved motor outcomes [429]. Piantino and co-workers also reported that the implantation of the composite PEG-PLA (PEG-poly-lactic acid) hydrogel containing NT3 improved BBB score and ladder walker performance, and increased the number of axons [432].

In another study, Teng and colleagues reported that the combination of NSCs and synthetic **PLGA** (poly-lactic-co-glycolic acid) scaffolds with aligned porosity reduce scar formation and improved functional recovery [433].

A synthetic matrix composed by peptide amphiphile (PA) molecules that self-assemble into cylindrical nanofibers and display bioactive epitopes, on their surfaces (**IKVAV-PA**) also demonstrated to improve motor function. Furthermore, IKVAV-PA nanofibers reduced astrogliosis, decreased cell death and promoted regeneration of ascending and descending track axons through the lesion site [434].

Several researchers prefer natural origins biomaterials rather than the synthetics ones. The main advantage of these materials is their potential bioactive behavior, without any further chemical adjustment. Some of them are also components of the human body, which greatly increase their capability to interact with the host's tissue. For instance, **collagen**, a major constituent of the ECM, has been used to fill the gap in transection models of SCI. Moreover, the NeuroGenTM Nerve Guide, a commercial product made from type I collagen, received FDA approval for marketing in 2001. After implantation in SCI rodents, collagen already demonstrated to increase the number axons either when used alone [435], in combination with NT3 [436] or even when used to fill PHEMA-MMA [427] and chitosan [437] tubes. These histologic observations were usually correlated with functional improvements. The use of collagens is, however, controversial, given that this molecule is a component of the glial scar.

The ECM extract from the Engelberth Holm Swarm sarcoma is also used as a natural 3D structure to support axon regeneration. This extract is commercial known as **Matrigel**

and contains several bioactive molecules, such as, laminin, fibronectin, and proteoglycans, with laminin predominating [438]. Lee and colleagues reported that the injection of matrigel combined with human NSCs in SCI dogs led to NSCs differentiation into neurons and oligodendrocytes, increased sensory axonal regeneration, and resulted in functional improvements [439]. In a recent study from Patel and co-workers, matrigel was combined with SCs and injected in a contusion rat model of SCI. This approach allowed the cell infiltration through the gel, increased angiogenesis and SCs survival, facilitated intraslesional axon growth, and promoted functional recovery both in overground locomotion and grid walk test [440].

Fibrin, the major constituent of the blood clots, is probably the most widely gel used in SCI. Fibrin contains several cell binding receptors and function as bridging molecule for many types of cells. It is usually used as a vehicle for cell transplantation or drug delivery, or even in conjugation with synthetic hydrogels [441]. Taylor and colleagues demonstrated that a fibrin gel engineered to release NT3 after degradation by invading cells, increased nervous cells infiltration and diminished the glial scar [442]. Moreover, the use of fibrin in combination with cAMP and pre-differentiated NSCs resulted in extensive axonal regeneration into the injury site of fully transected rats, and improved functional recovery after 6 weeks [443]. In addition to these applications, fibrin glue is regularly used for stabilization of cellular bridges to the implantation site [444, 445].

The oceans are an almost unlimited source of natural biomaterials for TE. **Agarose** and **Alginate** are two examples of polysaccharides derived from seaweed that were also employed as 3D constructs for SCI repair. Kim and colleagues injected an agarose hydrogel combined with PLGA nanoparticles and MP in a hemisection rat model of SCI

and reported a decrease on the lesion volume, macrophages invasion, proteoglycans content and oxidative stress. These histological outcomes were correlated with improved behavioral function [446]. Alternatively, Tobias and co-workers demonstrated that engineered-modified fibroblasts to produce BDNF encapsulated in alginate microcapsules can be delivery to the spinal cord without the need for immunosuppression. Moreover this constructs promoted growth of regenerating axons and improved the function of affected limbs [447].

Here we only report on biomaterials that improved both histological and behavioral outcomes. However, it is possible to find much more work in literature using different polymers that demonstrated to have some potential for SCI repair. A review published in May 2011 by Perale and colleagues identified 35 different hydrogels that aimed the repair of the spinal cord [416]. Nowadays the number is probably higher, and more will be described. It is important to note that many studies did not access or failed to demonstrate motor improvements after biomaterial implantation, and even the approaches that showed better functional outcomes needed more pre-clinical data in order to gain robustness (more information about TE in SCI can be found on Gilbert *et al.* review [448]).

One challenge for the clinical success of TE is the replication from independent labs of many of the approaches proposed until now. This is particular complicated because even the labs that use the same biomaterials, most of the time combined them with different cells or different bioactive molecules. With the possible combinations increasing the probability of independent labs used the same approaches decrease. Another challenge for TE is to start moving from transection to contusion/compression models of injury.

These models better mimic the clinical situation. However, they do represent higher challenge for scientists given that they lost the available space for scaffold implantation. In brief, TE owns great potential for SCI treatment; yet, more pre-clinical experiments are needed until moved to a clinical situation.

10. Final Remarks

Spinal cord injury (SCI) remains a frequent and devastating problem of modern society. Through a comprehension of essential pathophysiologic mechanisms involved in the evolution of SCI, treatments aimed at ameliorating neural damage were being developed. Although there are no fully restorative treatments for SCI, various rehabilitative, cellular and molecular therapies have been tested in animal models. Many of these have reached, or are approaching, clinical trials. However, until now none of them successfully treat the patients. Although utility of these therapeutic options provides modest benefits, there is a critical need to identify novel methodologies to treat or repair the injured spinal cord in hope to improve patient's quality of life. To stimulate CNS axons to regenerate, one essential repair strategy is to promote the neurons' intrinsic capacity to do so. Another effective method is to overcome the myelin and glial scar inhibition. Moreover, transplantation of tissues or cells, combined with permissive trophic factors, cell adhesion molecules and/or biomaterials, is essential for creating a growth-permissive topography to promote axonal regeneration across the injury site. It is now of general consensus that successful functional recovery will not simply rely on a single therapeutic approach.

Future clinical regenerative approaches will likely include combination of multiple strategies.

Challenges ahead comprise testing whether some of the most promising strategies in animal models are reproducible by different independent laboratories. Unfortunately, the facilities of research excellence in spinal cord injury (FORE-SCI) have been incapable to replicate studies from others laboratories. This inability to confirm potentially important outcomes with confidence has prevented new approaches from moving forward to clinical studies. The approaches that prove to be reliable also face the challenge of showing that the promising results in animal models are also beneficial for human patients suffering from SCI. However, it is imperative that translation studies be made in the most rigorous and informed fashion to determine safety and possible efficacy of the therapeutic approach. With this in mind, the International Campaign for Cures of Spinal Cord Injury Paralysis (ICCP) supported an international panel tasked with reviewing the methodology for clinical trials in spinal cord injury (SCI), and making recommendations on the conduct of future trials [449-452]. It is expected that these guidelines will provide a basis for the design of trials and for future revisions leading to continually improving generations of valid SCI clinical trial protocols.

References

- [1] Porter R. The Cambridge Illustrated History of Medicine. New York: Cambridge University Press; 1996.
- [2] Dobkin BH, Havton LA. Basic Advances and New Avenues in Therapy of Spinal Cord Injury. Annual Review of Medicine. 2004;55:255-82.
- [3] Vander AJ, Luciano DS, Sherman JH. Human Physiology: The Mechanisms of Body Function. Eighth ed: McGraw-Hill; 2001.
- [4] Purves D, Augustine G, Fitzpatrick D. Neuroscience. Third ed. Sunderland: Sinauer Associates 2004.
- [5] Van-De-Graaff. Human Anatomy. sixth ed: McGraw-Hill; 2001.
- [6] Mader S. Understanding Human Anatomy and Physiology. Fifth ed: McGraw-Hill Higher Education; 2004.
- [7] Salgado A, Silva, NA, Neves, NM, Reis RL. Hydrogel Based Systems for Spinal Cord Injury Regeneration. In: Reis R, editor. Natural-based polymers for biomedical applications. Cambridge: Woodhead Publishing Limited; 2008.
- [8] Watson C, Paxinos G, Kayalioglu G. Chapter 1 - The Organization of the Spinal Cord. In: Watson C, Paxinos G, Kayalioglu G, editors. The Spinal Cord. San Diego: Academic Press; 2009. p. 1-7.
- [9] DeVivo MJ, Go BK, Jackson AB. Overview of the National Spinal Cord Injury Statistical Center database. Journal of Spinal Cord Medicine. 2002;25:335-8.
- [10] Bunge RP, Puckett WR, Becerra JL, Marcillo A, Quencer RM. Observations on the pathology of human spinal cord injury. A review and classification of 22 new cases with details from a case of chronic cord compression with extensive focal demyelination. Advances in neurology. 1993;59:75-89.
- [11] Bunge RP, Puckett WR, Hiester ED. Observations on the pathology of several types of human spinal cord injury, with emphasis on the astrocyte response to penetrating injuries. Advances in neurology. 1997;72:305-15.
- [12] Cramer SC, Lastra L, Lacourse MG, Cohen MJ. Brain motor system function after chronic, complete spinal cord injury. Brain. 2005;128:2941-50.

- [13] Yiu G, He Z. Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci.* 2006;7:617-27.
- [14] Allen AR. Surgery of experimental lesions of spinal cord equivalent to crush injury of fracture dislocation of spinal column. A preliminary report. *J Am Med Assoc.* 1911;57:878-80.
- [15] Allen AR. Remarks on the histopathological changes in the spinal cord due to impact. An experimental study. *J Nerv Ment Dis.* 1914;41:141-7.
- [16] Bareyre FM, Schwab ME. Inflammation, degeneration and regeneration in the injured spinal cord: insights from DNA microarrays. *Trends in Neurosciences.* 2003;26:555-63.
- [17] Tator CH. Review of experimental spinal cord injury with emphasis on the local and systemic circulatory effects. *Neuro-Chirurgie.* 1991;37:291-302.
- [18] Tator CH, Fehlings MG. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. *Journal of Neurosurgery.* 1991;75:15-26.
- [19] Toborek M, Malecki A, Garrido R, Mattson MP, Hennig B, Young B. Arachidonic Acid-Induced Oxidative Injury to Cultured Spinal Cord Neurons. *Journal of Neurochemistry.* 1999;73:684-92.
- [20] Sandler AN, Tator CH. Review of the effect of spinal cord trauma on the vessels and blood flow in the spinal cord. *Journal of Neurosurgery.* 1976;45:638-46.
- [21] Goodman JH, Bingham WG, Jr, Hunt WE. Platelet Aggregation in Experimental Spinal Cord Injury: Ultrastructural Observations. *Arch Neurol.* 1979;36:197-201.
- [22] Chan PH, Fishman RA. The role of arachidonic acid in vasogenic brain edema. *Federation proceedings.* 1984;43:210-3.
- [23] Banik NL, Hogan EL, Powers JM, Whetstone LJ. Degradation of cytoskeletal proteins in experimental spinal cord injury. *Neurochemical Research.* 1982;7:1465-75.
- [24] Stys PK. Anoxic and Ischemic Injury of Myelinated Axons in CNS White Matter: From Mechanistic Concepts to Therapeutics. *J Cereb Blood Flow Metab.* 1998;18:2-25.
- [25] Xu GY, McAdoo DJ, Hughes MG, Robak G, de Castro Jr R. Considerations in the determination by microdialysis of resting extracellular amino acid concentrations and release upon spinal cord injury. *Neuroscience.* 1998;86:1011-21.

- [26] McAdoo DJ, Xu G-Y, Robak G, Hughes MG. Changes in Amino Acid Concentrations over Time and Space around an Impact Injury and Their Diffusion Through the Rat Spinal Cord. *Experimental Neurology*. 1999;159:538-44.
- [27] Liu D, Xu GY, Pan E, McAdoo DJ. Neurotoxicity of glutamate at the concentration released upon spinal cord injury. *Neuroscience*. 1999;93:1383-9.
- [28] Farooque M, Hillered L, Holtz A, Olsson Y. Changes of Extracellular Levels of Amino Acids after Graded Compression Trauma to the Spinal Cord: An Experimental Study in the Rat Using Microdialysis. *Journal of Neurotrauma*. 1996;13:537-48.
- [29] Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Crowe MS. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nat Med*. 1997;3:73-6.
- [30] Popovich PG, Wei P, Stokes BT. Cellular inflammatory response after spinal cord injury in sprague-dawley and lewis rats. *The Journal of Comparative Neurology*. 1997;377:443-64.
- [31] Mabon PJ, Weaver LC, Dekaban GA. Inhibition of Monocyte/Macrophage Migration to a Spinal Cord Injury Site by an Antibody to the Integrin α D: A Potential New Anti-inflammatory Treatment. *Experimental Neurology*. 2000;166:52-64.
- [32] Means ED, Anderson DK. Neuronophagia by Leukocytes in Experimental Spinal Cord Injury. *Journal of Neuropathology and Experimental Neurology*. 1983;42:707-19.
- [33] Taoka Y, Okajima K, Uchiba M, Murakami K, Kushimoto S, Johnno M, et al. Role of neutrophils in spinal cord injury in the rat. *Neuroscience*. 1997;79:1177-82.
- [34] Donnelly DJ, Popovich PG. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Experimental Neurology*. 2008;209:378-88.
- [35] Merrill J, Ignarro L, Sherman M, Melinek J, Lane T. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *The Journal of Immunology*. 1993;151:2132-41.
- [36] Xu J, Fan G, Chen S, Wu Y, Xu XM, Hsu CY. Methylprednisolone inhibition of TNF- α expression and NF-kB activation after spinal cord injury in rats. *Molecular Brain Research*. 1998;59:135-42.

- [37] White RE, Yin FQ, Jakeman LB. TGF- α increases astrocyte invasion and promotes axonal growth into the lesion following spinal cord injury in mice. *Experimental Neurology*. 2008;214:10-24.
- [38] Jones LL, Margolis RU, Tuszynski MH. The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. *Experimental Neurology*. 2003;182:399-411.
- [39] Gallo V, Bertolotto A, Levi G. The proteoglycan chondroitin sulfate is present in a subpopulation of cultured astrocytes and in their precursors. *Developmental Biology*. 1987;123:282-5.
- [40] Katoh-Semba R, Matsuda M, Kato K, Oohira A. Chondroitin Sulphate Proteoglycans in the Rat Brain: Candidates for Axon Barriers of Sensory Neurons and the Possible Modification by Laminin of their Actions. *European Journal of Neuroscience*. 1995;7:613-21.
- [41] Kennedy P, Rogers BA. Anxiety and depression after spinal cord injury: A longitudinal analysis. *Archives of Physical Medicine and Rehabilitation*. 2000;81:932-7.
- [42] Stormer S, Gerner HJ, Gruninger W, Metzmacher K, Follinger S, Wienke C, et al. Chronic pain/dysaesthesiae in spinal cord injury patients: results of a multicentre study. *Spinal cord*. 1997;35:446-55.
- [43] Martin E S. Nogo and axon regeneration. *Current Opinion in Neurobiology*. 2004;14:118-24.
- [44] Simonen M, Pedersen V, Weinmann O, Schnell L, Buss A, Ledermann B, et al. Systemic Deletion of the Myelin-Associated Outgrowth Inhibitor Nogo-A Improves Regenerative and Plastic Responses after Spinal Cord Injury. *Neuron*. 2003;38:201-11.
- [45] Domeniconi M, Cao Z, Spencer T, Sivasankaran R, Wang KC, Nikulina E, et al. Myelin-Associated Glycoprotein Interacts with the Nogo66 Receptor to Inhibit Neurite Outgrowth. *Neuron*. 2002;35:283-90.
- [46] Fouad K, Dietz V, Schwab ME. Improving axonal growth and functional recovery after experimental spinal cord injury by neutralizing myelin associated inhibitors. *Brain Research Reviews*. 2001;36:204-12.

- [47] Li M, Shibata A, Li C, Braun PE, McKerracher L, Roder J, et al. Myelin-associated glycoprotein inhibits neurite/axon growth and causes growth cone collapse. *Journal of Neuroscience Research*. 1996;46:404-14.
- [48] Milev P, Friedlander DR, Sakurai T, Karthikeyan L, Flad M, Margolis RK, et al. Interactions of the chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. *The Journal of Cell Biology*. 1994;127:1703-15.
- [49] Pesheva P, Gennarini G, Goridis C, Schachner M. The F3/11 cell adhesion molecule mediates the repulsion of neurons by the extracellular matrix glycoprotein J1-160/180. *Neuron*. 1993;10:69-82.
- [50] Apostolova I, Irintchev A, Schachner M. Tenascin-R Restricts Posttraumatic Remodeling of Motoneuron Innervation and Functional Recovery after Spinal Cord Injury in Adult Mice. *The Journal of Neuroscience*. 2006;26:7849-59.
- [51] Johnson-Green PC, Dow KE, Riopelle RJ. Characterization of glycosaminoglycans produced by primary astrocytes in vitro. *Glia*. 1991;4:314-21.
- [52] Fawcett JW, Asher RA. The glial scar and central nervous system repair. *Brain Research Bulletin*. 1999;49:377-91.
- [53] McKeon RJ, Höke A, Silver J. Injury-Induced Proteoglycans Inhibit the Potential for Laminin-Mediated Axon Growth on Astrocytic Scars. *Experimental Neurology*. 1995;136:32-43.
- [54] Levine J. Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury. *The Journal of Neuroscience*. 1994;14:4716-30.
- [55] Geisert Jr EE, Bidanset DJ, Del Mar N, Robson JA. Up-Regulation of a Keratan Sulfate Proteoglycan Following Cortical Injury in Neonatal Rats. *International Journal of Developmental Neuroscience*. 1996;14:257-67.
- [56] Davies SJA, Fitch MT, Memberg SP, Hall AK, Raisman G, Silver J. Regeneration of adult axons in white matter tracts of the central nervous system. *Nature*. 1997;390:680-3.
- [57] Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci*. 2004;5:146-56.
- [58] Busch SA, Silver J. The role of extracellular matrix in CNS regeneration. *Current Opinion in Neurobiology*. 2007;17:120-7.

- [59] Menet V, Prieto M, Privat A, Ribotta MGy. Axonal plasticity and functional recovery after spinal cord injury in mice deficient in both glial fibrillary acidic protein and vimentin genes. *Proceedings of the National Academy of Sciences*. 2003;100:8999-9004.
- [60] Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV. Reactive Astrocytes Protect Tissue and Preserve Function after Spinal Cord Injury. *The Journal of Neuroscience*. 2004;24:2143-55.
- [61] Okada S, Nakamura M, Katoh H, Miyao T, Shimazaki T, Ishii K, et al. Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med*. 2006;12:829-34.
- [62] Qi H-X, Stepniewska I, Kaas JH. Reorganization of Primary Motor Cortex in Adult Macaque Monkeys With Long-Standing Amputations. *Journal of Neurophysiology*. 2000;84:2133-47.
- [63] Bruehlmeier M, Dietz V, Leenders KL, Roelcke U, Missimer J, Curt A. How does the human brain deal with a spinal cord injury? *European Journal of Neuroscience*. 1998;10:3918-22.
- [64] Levy Jr WJ, Amassian VE, Traad M, Cadwell J. Focal magnetic coil stimulation reveals motor cortical system reorganized in humans after traumatic quadriplegia. *Brain Research*. 1990;510:130-4.
- [65] Topka H, Cohen LG, Cole RA, Hallett M. Reorganization of corticospinal pathways following spinal cord injury. *Neurology*. 1991;41:1276.
- [66] Jain N, Catania KC, Kaas JH. Deactivation and reactivation of somatosensory cortex after dorsal spinal cord injury. *Nature*. 1997;386:495-8.
- [67] Lawrence DG, Kuypers HGJ. Functional Organization of Motor System in Monkey. *Brain*. 1968;91.
- [68] Lawrence DG, Kuypers HGJ. Functional Organization of Motor System in Monkey 2. Effects of Lesions of Descending Brain-Stem Pathways. *Brain*. 1968;91:15.
- [69] Kaas JH, Qi H-X, Burish MJ, Gharbawie OA, Onifer SM, Massey JM. Cortical and subcortical plasticity in the brains of humans, primates, and rats after damage to sensory afferents in the dorsal columns of the spinal cord. *Experimental Neurology*. 2008;209:407-16.

- [70] Rossignol S, Drew T, Brustein E, Jiang W. Chapter 31 Locomotor Performance and Adaptation after Partial or Complete Spinal Cord Lesions in the Cat. In: Binder MD, editor. *Progress in Brain Research*: Elsevier; 1999. p. 349-65.
- [71] de Leon RD, Hodgson JA, Roy RR, Edgerton VR. Locomotor Capacity Attributable to Step Training Versus Spontaneous Recovery After Spinalization in Adult Cats. *Journal of Neurophysiology*. 1998;79:1329-40.
- [72] Wernig A, Müller S, Nanassy A, Cagol E. Laufband Therapy Based on 'Rules of Spinal Locomotion' is Effective in Spinal Cord Injured Persons. *European Journal of Neuroscience*. 1995;7:823-9.
- [73] Ballermann M, Fouad K. Spontaneous locomotor recovery in spinal cord injured rats is accompanied by anatomical plasticity of reticulospinal fibers. *European Journal of Neuroscience*. 2006;23:1988-96.
- [74] Courtine G, Song B, Roy RR, Zhong H, Herrmann JE, Ao Y, et al. Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. *Nat Med*. 2008;14:69-74.
- [75] Boulenguez P, Liabeuf S, Bos R, Bras H, Jean-Xavier C, Brocard C, et al. Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. *Nat Med*. 2010;16:302-7.
- [76] Loy DN, Magnuson DSK, Zhang YP, Onifer SM, Mills MD, Cao Q-l, et al. Functional Redundancy of Ventral Spinal Locomotor Pathways. *The Journal of Neuroscience*. 2002;22:315-23.
- [77] Jones EG. GABAergic Neurons and Their Role in Cortical Plasticity in Primates. *Cerebral Cortex*. 1993;3:361-72.
- [78] Jacobs K, Donoghue J. Reshaping the cortical motor map by unmasking latent intracortical connections. *Science*. 1991;251:944-7.
- [79] Hendry SHC, Jones EG. Reduction in number of immunostained GABAergic neurones in deprived-eye dominance columns of monkey area 17. *Nature*. 1986;320:750-3.
- [80] Roy FD, Zewdie ET, Gorassini MA. Short-interval intracortical inhibition with incomplete spinal cord injury. *Clinical Neurophysiology*. 2011;122:1387-95.

- [81] Bracken MB, Holford TR. Neurological and functional status 1 year after acute spinal cord injury: estimates of functional recovery in national Acute Spinal Cord Injury Study II from results modeled in National Acute Spinal Cord Injury Study III. *Journal of Neurosurgery*. 2002;96:259-66.
- [82] Carlson GD, Minato Y, Okada A, Gorden CD, Warden KE, Barbeau JM, et al. Early Time-Dependent Decompression for Spinal Cord Injury: Vascular Mechanisms of Recovery. *Journal of Neurotrauma*. 1997;14:951-62.
- [83] Dimar JRI, Glassman SD, Raque GH, Zhang YP, Shields CB. The Influence of Spinal Canal Narrowing and Timing of Decompression on Neurologic Recovery After Spinal Cord Contusion in a Rat Model. *Spine*. 1999;24:1623.
- [84] Dolan EJ, Tator CH, Endrenyi L. The value of decompression for acute experimental spinal cord compression injury. *Journal of Neurosurgery*. 1980;53:749-55.
- [85] Brodkey JS, Richards DE, Blasingame JP, Nulsen FE. Reversible spinal cord trauma in cats. *Journal of Neurosurgery*. 1972;37:591-3.
- [86] Wilmot CB, Hall KM. Evaluation of the acute management of tetraplegia: conservative versus surgical treatment. *Paraplegia*. 1986;24:148-53.
- [87] Marshall LF, Knowlton S, Garfin SR, Klauber MR, Eisenberg HM, Kopaniky D, et al. Deterioration following spinal cord injury. *Journal of Neurosurgery*. 1987;66:400-4.
- [88] Bedbrook GM, Sakae T. A review of cervical spine injuries with neurological dysfunction. *Paraplegia*. 1982;20:321-33.
- [89] Fehlings MG, Perrin RG. The role and timing of early decompression for cervical spinal cord injury: Update with a review of recent clinical evidence. *Injury*. 2005;36:S13-S26.
- [90] Hall ED, Braughler JM. Effects of intravenous methylprednisolone on spinal cord lipid peroxidation and (Na⁺ + K⁺)-ATPase activity. *Journal of Neurosurgery*. 1982;57:247-53.
- [91] Tator CH. Biology of Neurological Recovery and Functional Restoration after Spinal Cord Injury. *Neurosurgery*. 1998;42:696-707.
- [92] Hall ED, Braughler JM. Acute effects of intravenous glucocorticoid pretreatment on the in vitro peroxidation of cat spinal cord tissue. *Experimental Neurology*. 1981;73:321-4.

- [93] Bracken MB, Collins WF, Freeman DF, Shepard MJ, Wagner FW, Silten RM, et al. Efficacy of Methylprednisolone in Acute Spinal Cord Injury. *JAMA: The Journal of the American Medical Association*. 1984;251:45-52.
- [94] Amar AP, Levy ML. Pathogenesis and Pharmacological Strategies for Mitigating Secondary Damage in Acute Spinal Cord Injury. *Neurosurgery*. 1999;44:1027-39.
- [95] Braughler JM, Hall ED. Effects of multi-dose methylprednisolone sodium succinate administration on injured cat spinal cord neurofilament degradation and energy metabolism. *Journal of Neurosurgery*. 1984;61:290-5.
- [96] Bracken MB, Shepard MJ, Collins WF, Holford TR, Young W, Baskin DS, et al. A Randomized, Controlled Trial of Methylprednisolone or Naloxone in the Treatment of Acute Spinal-Cord Injury. *New England Journal of Medicine*. 1990;322:1405-11.
- [97] Hurlbert RJ. Methylprednisolone for acute spinal cord injury: an inappropriate standard of care*. *Journal of Neurosurgery: Spine*. 2000;93:1-7.
- [98] Hanigan WC, Anderson RJ. Commentary on NASCIS-2. *Journal of Spinal Disorders & Techniques*. 1992;5:125-31.
- [99] Coleman WP, Benzel E, Cahill DW, Ducker T, Geisler F, Green B, et al. A Critical Appraisal of the Reporting of the National Acute Spinal Cord Injury Studies (II and III) of Methylprednisolone in Acute Spinal Cord Injury. *Journal of Spinal Disorders & Techniques*. 2000;13:185-99.
- [100] Short D. Use of steroids for acute spinal cord injury must be reassessed. *BMJ*. 2000;321:1224.
- [101] Short DJ, El Masry WS, Jones PW. High dose methylprednisolone in the management of acute spinal cord injury - a systematic review from a clinical perspective. *Spinal cord*. 2000;38:273-86.
- [102] Bracken MB, Shepard MJ, Holford TR, Leo-Summers L, Aldrich EF, Fazl M, et al. Administration of Methylprednisolone for 24 or 48 Hours or Tirilazad Mesylate for 48 Hours in the Treatment of Acute Spinal Cord Injury. *JAMA: The Journal of the American Medical Association*. 1997;277:1597-604.
- [103] Bracken MB, Shepard MJ, Holford TR, Leo-Summers L, Aldrich EF, Fazl M, et al. Methylprednisolone or tirilazad mesylate administration after acute spinal cord injury: 1-year follow up. *Journal of Neurosurgery*. 1998;89:699-706.

- [104] AANS/CNS. Pharmacological Therapy after Acute Cervical Spinal Cord Injury. *Neurosurgery*. 2002;50:S63-S72.
- [105] Hurlbert RJ, Hamilton MG. Methylprednisolone for acute spinal cord injury: 5-year practice reversal. *The Canadian journal of neurological sciences Le journal canadien des sciences neurologiques*. 2008;35:41-5.
- [106] Cerqueira S, Silva B, Oliveira J, Mano J, Sousa N, Salgado A, et al. Multifunctionalized CMCh/PAMAM Dendrimer Nanoparticles Modulate the Cellular Uptake by Astrocytes and Oligodendrocytes in Primary Cultures of Glial Cells,. *Macromolecular Bioscience*. 2012;accepted for publication.
- [107] Cerqueira S, Oliveira J, Mano J, Sousa N, Salgado A, Reis RL. In Vitro Modulation of Microglial Cells Viability by Nanoparticle-Mediated Intracellular Release of Methylprednisolone, submitted.
- [108] Hawryluk GWJ, Rowland J, Kwon BK, Fehlings MG. Protection and repair of the injured spinal cord: a review of completed, ongoing, and planned clinical trials for acute spinal cord injury. *Neurosurgical Focus*. 2008;25:E14.
- [109] Blight AR. Cellular morphology of chronic spinal cord injury in the cat: Analysis of myelinated axons by line-sampling. *Neuroscience*. 1983;10:521-43.
- [110] Blight AR, Toombs JP, Bauer MS, Widmer WR. The Effects of 4-Aminopyridine on Neurological Deficits in Chronic Cases of Traumatic Spinal Cord Injury in Dogs: A Phase I Clinical Trial. *Journal of Neurotrauma*. 1991;8:103-19.
- [111] Modi HN, Suh S-W, Hong J-Y, Yang J-H. The Effects of Spinal Cord Injury Induced by Shortening on Motor Evoked Potentials and Spinal Cord Blood Flow. *Journal of Bone and Joint Surgery-American Volume*. 2011;93A:1781-9.
- [112] Wells JEA, Hurlbert RJ, Fehlings MG, Yong VW. Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. *Brain*. 2003;126:1628-37.
- [113] Jakeman L, Ma M, Stokes B. Considering the use of transgenic mice in spinal cord research. In: Marwah J, Dixon C, Banik N, editors. *Traumatic CNS injury*. Scottsdale: Prominent Press; 2001. p. 180–201.
- [114] Rosenzweig ES, McDonald JW. Rodent models for treatment of spinal cord injury: research trends and progress toward useful repair. *Current Opinion in Neurology*. 2004;17:121-31.

- [115] Gruner JA. A Monitored Contusion Model of Spinal-Cord Injury in the Rat. *Journal of Neurotrauma*. 1992;9:123-8.
- [116] Behrmann DL, Bresnahan JC, Beattie MS, Shah BR. Spinal Cord Injury Produced by Consistent Mechanical Displacement of the Cord in Rats: Behavioral and Histologic Analysis. *Journal of Neurotrauma*. 1992;9:197-217.
- [117] Anderson T, Stokes B. Experimental models for spinal cord injury research: physical and physiological considerations. Larchmont, NY, USA: Liebert; 1992.
- [118] Rivlin AS, Tator CH. Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat. *Surgical neurology*. 1978;10:38-43.
- [119] Borgens RB, Shi R. Immediate recovery from spinal cord injury through molecular repair of nerve membranes with polyethylene glycol. *The FASEB Journal*. 2000;14:27-35.
- [120] Vanický I, Urdžíková L, Saganová K, Čížková D, Gálik J. A Simple and Reproducible Model of Spinal Cord Injury Induced by Epidural Balloon Inflation in the Rat. *Journal of Neurotrauma*. 2001;18:1399-407.
- [121] Fehlings MG, Tator CH. The relationships among the severity of spinal cord injury, residual neurological function, axon counts, and counts of retrogradely labeled neurons after experimental spinal cord injury. *Experimental Neurology*. 1995;132:220-8.
- [122] Fehlings MG, Tator CH, Linden RD. The relationships among the severity of spinal cord injury, motor and somatosensory evoked potentials and spinal cord blood flow. *Electroencephalography and Clinical Neurophysiology/Evoked Potentials Section*. 1989;74:241-59.
- [123] Guha A, Tator CH, Endrenyi L, Piper I. Decompression of the spinal cord improves recovery after acute experimental spinal cord compression injury. *Paraplegia*. 1987;25:324-39.
- [124] Schwartz G, Fehlings MG. Evaluation of the neuroprotective effects of sodium channel blockers after spinal cord injury: improved behavioral and neuroanatomical recovery with riluzole. *Journal of Neurosurgery*. 2001;94:245-56.
- [125] Basso DM. Behavioral Testing After Spinal Cord Injury: Congruities, Complexities, and Controversies. *Journal of Neurotrauma*. 2004;21:395-404.

- [126] Basso DM, Beattie MS, Bresnahan JC. A Sensitive and Reliable Locomotor Rating-Scale for Open-Field Testing in Rats. *Journal of Neurotrauma*. 1995;12:1-21.
- [127] Brustein E, Rossignol S. Recovery of Locomotion After Ventral and Ventrolateral Spinal Lesions in the Cat. I. Deficits and Adaptive Mechanisms. *Journal of Neurophysiology*. 1998;80:1245-67.
- [128] Popovich PG, Guan Z, Wei P, Huitinga I, van Rooijen N, Stokes BT. Depletion of Hematogenous Macrophages Promotes Partial Hindlimb Recovery and Neuroanatomical Repair after Experimental Spinal Cord Injury. *Experimental Neurology*. 1999;158:351-65.
- [129] Goldberger ME, Bregman BS, Vierck CJ, Brown M. Criteria for assessing recovery of function after spinal cord injury: behavioral methods. *Experimental Neurology*. 1990;107:113-7.
- [130] Armstrong DM. The supraspinal control of mammalian locomotion. *The Journal of physiology*. 1988;405:1-37.
- [131] Broton JG, Nikolic Z, Suys S, Calancie B. Kinematic Analysis of Limb Position during Quadrupedal Locomotion in Rats. *Journal of Neurotrauma*. 1996;13:409-16.
- [132] Metz GAS, Merkler D, Dietz V, Schwab ME, Fouad K. Efficient testing of motor function in spinal cord injured rats. *Brain Research*. 2000;883:165-77.
- [133] Lankhorst AJ, Verzijl MR, Hamers FPT. Experimental spinal cord contusion injury: Comparison of different outcome parameters. *Neuroscience Research Communications*. 1999;24:135-48.
- [134] Metz GA, Whishaw IQ. Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. *Journal of Neuroscience Methods*. 2002;115:169-79.
- [135] Chan CCM, Khodarahmi K, Liu J, Sutherland D, Oschipok LW, Steeves JD, et al. Dose-dependent beneficial and detrimental effects of ROCK inhibitor Y27632 on axonal sprouting and functional recovery after rat spinal cord injury. *Experimental Neurology*. 2005;196:352-64.
- [136] Zorner B, Filli L, Starkey ML, Gonzenbach R, Kasper H, Rothlisberger M, et al. Profiling locomotor recovery: comprehensive quantification of impairments after CNS damage in rodents. *Nat Meth*. 2010;7:701-8.

- [137] Ichiyama RM, Courtine G, Gerasimenko YP, Yang GJ, van den Brand R, Lavrov IA, et al. Step Training Reinforces Specific Spinal Locomotor Circuitry in Adult Spinal Rats. *The Journal of Neuroscience*. 2008;28:7370-5.
- [138] Gale K, Kerasidis H, Wrathall JR. Spinal cord contusion in the rat: Behavioral analysis of functional neurologic impairment. *Experimental Neurology*. 1985;88:123-34.
- [139] Syková E, Jendelová P, Urdziková L, Lesný P, Hejčl A. Bone Marrow Stem Cells and Polymer Hydrogels—Two Strategies for Spinal Cord Injury Repair. *Cellular and Molecular Neurobiology*. 2006;26:1111-27.
- [140] Gris D, Marsh DR, Oatway MA, Chen Y, Hamilton EF, Dekaban GA, et al. Transient Blockade of the CD11d/CD18 Integrin Reduces Secondary Damage after Spinal Cord Injury, Improving Sensory, Autonomic, and Motor Function. *The Journal of Neuroscience*. 2004;24:4043-51.
- [141] Šedý J, Urdziková L, Jendelová P, Syková E. Methods for behavioral testing of spinal cord injured rats. *Neuroscience & Biobehavioral Reviews*. 2008;32:550-80.
- [142] Falck B, Hillarp NA, Thieme G, Torp A. Fluorescence of Catechol Amines and Related-Compounds Condensed With Formaldehyde. *Brain Research Bulletin*. 1982;9:R11-R5.
- [143] W.Maxwell C. The Emergence of Modern Neuroanatomy and Developmental Neurobiology. *Neuron*. 1998;20:413-26.
- [144] Moore RY, Loy R. Fluorescence histochemistry. In: Robertson R, editor. *Neuroanatomical Research Techniques*. New York: Academic Press; 1978. p. 115–39.
- [145] Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. *Nat Neurosci*. 2004;7:269-77.
- [146] Xu XM, Chen A, Guenard V, Kleitman N, Bunge MB. Bridging Schwann cell transplants promote axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. *Journal of Neurocytology*. 1997;26:1-16.
- [147] GrandPre T, Li S, Strittmatter SM. Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature*. 2002;417:547-51.

- [148] Liu K, Lu Y, Lee JK, Samara R, Willenberg R, Sears-Kraxberger I, et al. PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nat Neurosci.* 2010;13:1075-81.
- [149] Wong L-F, Yip PK, Battaglia A, Grist J, Corcoran J, Maden M, et al. Retinoic acid receptor [beta]2 promotes functional regeneration of sensory axons in the spinal cord. *Nat Neurosci.* 2006;9:243-50.
- [150] Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, et al. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2002;99:3024-9.
- [151] Kaneko S, Iwanami A, Nakamura M, Kishino A, Kikuchi K, Shibata S, et al. A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord. *Nat Med.* 2006;12:1380-9.
- [152] Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Morshead CM, Fehlings MG. Delayed Transplantation of Adult Neural Precursor Cells Promotes Remyelination and Functional Neurological Recovery after Spinal Cord Injury. *The Journal of Neuroscience.* 2006;26:3377-89.
- [153] Rooney GE, McMahon SS, Ritter T, Garcia Y, Moran C, Madigan NN, et al. Neurotrophic Factor–Expressing Mesenchymal Stem Cells Survive Transplantation into the Contused Spinal Cord Without Differentiating into Neural Cells. *Tissue Engineering Part A.* 2009;15:3049-59.
- [154] Shen Y, Tenney AP, Busch SA, Horn KP, Cuascut FX, Liu K, et al. PTP σ Is a Receptor for Chondroitin Sulfate Proteoglycan, an Inhibitor of Neural Regeneration. *Science.* 2009;326:592-6.
- [155] Wang R, King T, Ossipov MH, Rossomando AJ, Vanderah TW, Harvey P, et al. Persistent restoration of sensory function by immediate or delayed systemic artemin after dorsal root injury. *Nat Neurosci.* 2008;11:488-96.
- [156] Detloff MR, Fisher LC, McGaughy V, Longbrake EE, Popovich PG, Basso DM. Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. *Experimental Neurology.* 2008;212:337-47.

- [157] Woerly S, Doan vD, Sosa N, de Vellis J, Espinosa A. Reconstruction of the transected cat spinal cord following NeuroGel™ implantation: axonal tracing, immunohistochemical and ultrastructural studies. *International Journal of Developmental Neuroscience*. 2001;19:63-83.
- [158] Wang X, Arcuino G, Takano T, Lin J, Peng WG, Wan P, et al. P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med*. 2004;10:821-7.
- [159] Ozdinler PH, Macklis JD. IGF-I specifically enhances axon outgrowth of corticospinal motor neurons. *Nat Neurosci*. 2006;9:1371-81.
- [160] Veeravalli KK, Dasari VR, Tsung AJ, Dinh DH, Gujrati M, Fassett D, et al. Human umbilical cord blood stem cells upregulate matrix metalloproteinase-2 in rats after spinal cord injury. *Neurobiology of Disease*. 2009;36:200-12.
- [161] Bradbury EJ, Moon LDF, Popat RJ, King VR, Bennett GS, Patel PN, et al. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature*. 2002;416:636-40.
- [162] Gaviria M, Privat A, d'Arbigny P, Kamenka J-M, Haton H, Ohanna F. Neuroprotective effects of a novel NMDA antagonist, Gacyclidine, after experimental contusive spinal cord injury in adult rats. *Brain Research*. 2000;874:200-9.
- [163] Imaizumi T, Lankford KL, Kocsis JD. Transplantation of olfactory ensheathing cells or Schwann cells restores rapid and secure conduction across the transected spinal cord. *Brain Research*. 2000;854:70-8.
- [164] Pinzon A, Calancie B, Oudega M, Noga BR. Conduction of impulses by axons regenerated in a Schwann cell graft in the transected adult rat thoracic spinal cord. *Journal of Neuroscience Research*. 2001;64:533-41.
- [165] Agrawal SK, Fehlings MG. Mechanisms of secondary injury to spinal cord axons in vitro: role of Na⁺, Na⁽⁺⁾-K⁽⁺⁾-ATPase, the Na⁽⁺⁾-H⁺ exchanger, and the Na⁽⁺⁾-Ca²⁺ exchanger. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1996;16:545-52.
- [166] Agrawal SK, Fehlings MG. Role of NMDA and Non-NMDA Ionotropic Glutamate Receptors in Traumatic Spinal Cord Axonal Injury. *The Journal of Neuroscience*. 1997;17:1055-63.

- [167] Gorska T, Majczynski H, Zmyslowski W. Overground locomotion in intact rats: contact electrode recording. *Acta Neurobiologiae Experimentalis*. 1998;58:227-37.
- [168] Mathé G, Jammet H, Pendie N. Transfusions et greffes de moelle osseuse homologue chez des humaine irradiées a haute dose accidentellement. *Nouvelle rev franc hematol*. 1959;4.
- [169] Gage FH. Mammalian Neural Stem Cells. *Science*. 2000;287:1433-8.
- [170] Lu P, Jones LL, Snyder EY, Tuszynski MH. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Experimental Neurology*. 2003;181:115-29.
- [171] Cao Q-l, Zhang YP, Howard RM, Walters WM, Tsoulfas P, Whittemore SR. Pluripotent Stem Cells Engrafted into the Normal or Lesioned Adult Rat Spinal Cord Are Restricted to a Glial Lineage. *Experimental Neurology*. 2001;167:48-58.
- [172] Shihabuddin LS, Horner PJ, Ray J, Gage FH. Adult Spinal Cord Stem Cells Generate Neurons after Transplantation in the Adult Dentate Gyrus. *The Journal of Neuroscience*. 2000;20:8727-35.
- [173] Bottai D, Madaschi L, Di Giulio AM, Gorio A. Viability-dependent promoting action of adult neural precursors in spinal cord injury. *Molecular medicine (Cambridge, Mass)*. 2008;14:634-44.
- [174] Hofstetter CP, Holmstrom NAV, Lilja JA, Schweinhardt P, Hao J, Spenger C, et al. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nat Neurosci*. 2005;8:346-53.
- [175] Okada S, Ishii K, Yamane J, Iwanami A, Ikegami T, Katoh H, et al. In vivo imaging of engrafted neural stem cells: its application in evaluating the optimal timing of transplantation for spinal cord injury. *The FASEB Journal*. 2005;19:1839-41.
- [176] Parr AM, Kulbatski I, Zahir T, Wang X, Yue C, Keating A, et al. Transplanted adult spinal cord-derived neural stem/progenitor cells promote early functional recovery after rat spinal cord injury. *Neuroscience*. 2008;155:760-70.
- [177] Lepore AC, Fischer I. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. *Experimental Neurology*. 2005;194:230-42.

- [178] Mitsui T, Shumsky JS, Lepore AC, Murray M, Fischer I. Transplantation of Neuronal and Glial Restricted Precursors into Contused Spinal Cord Improves Bladder and Motor Functions, Decreases Thermal Hypersensitivity, and Modifies Intraspinal Circuitry. *The Journal of Neuroscience*. 2005;25:9624-36.
- [179] Fehlings M, Vawda R. Cellular Treatments for Spinal Cord Injury: The Time is Right for Clinical Trials. *Neurotherapeutics*. 2011;8:704-20.
- [180] Brazelton TR, Rossi FMV, Keshet GI, Blau HM. From Marrow to Brain: Expression of Neuronal Phenotypes in Adult Mice. *Science*. 2000;290:1775-9.
- [181] Mezey É, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning Blood into Brain: Cells Bearing Neuronal Antigens Generated in Vivo from Bone Marrow. *Science*. 2000;290:1779-82.
- [182] Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *Journal of Neuroscience Research*. 2000;61:364-70.
- [183] Castro RF, Jackson KA, Goodell MA, Robertson CS, Liu H, Shine HD. Failure of Bone Marrow Cells to Transdifferentiate into Neural Cells in Vivo. *Science*. 2002;297:1299.
- [184] Wurmser AE, Gage FH. Stem cells: Cell fusion causes confusion. *Nature*. 2002;416:485-7.
- [185] Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature*. 2002;416:542-5.
- [186] Tetzlaff W, Okon EB, Karimi-Abdolrezaee S, Hill CE, Sparling JS, Plemel JR, et al. A Systematic Review of Cellular Transplantation Therapies for Spinal Cord Injury. *Journal of Neurotrauma*. 2010;28:1611-82.
- [187] Ohta M, Suzuki Y, Noda T, Ejiri Y, Dezawa M, Kataoka K, et al. Bone marrow stromal cells infused into the cerebrospinal fluid promote functional recovery of the injured rat spinal cord with reduced cavity formation. *Experimental Neurology*. 2004;187:266-78.
- [188] Urdžíková L, Jendelová P, Glogarová K, Burian M, Hájek M, Syková E. Transplantation of Bone Marrow Stem Cells as well as Mobilization by Granulocyte-

Colony Stimulating Factor Promotes Recovery after Spinal Cord Injury in Rats. *Journal of Neurotrauma*. 2006;23:1379-91.

[189] Deng Y, Liu Y, Zhu W, Bi X, Wang Y, Ye M, et al. The co-transplantation of human bone marrow stromal cells and embryo olfactory ensheathing cells as a new approach to treat spinal cord injury in a rat model. *Cytherapy*. 2008;10:551-64.

[190] Zurita M, Vaquero J. Bone marrow stromal cells can achieve cure of chronic paraplegic rats: Functional and morphological outcome one year after transplantation. *Neuroscience Letters*. 2006;402:51-6.

[191] Ankeny DP, McTigue DM, Jakeman LB. Bone marrow transplants provide tissue protection and directional guidance for axons after contusive spinal cord injury in rats. *Experimental Neurology*. 2004;190:17-31.

[192] Ribeiro CA, Salgado AJ, Fraga JS, Silva NA, Reis RL, Sousa N. The secretome of bone marrow mesenchymal stem cells-conditioned media varies with time and drives a distinct effect on mature neurons and glial cells (primary cultures). *Journal of Tissue Engineering and Regenerative Medicine*. 2011;5:668-72.

[193] Sykov, Eva, Homola A, Mazanec R, Lachmann H, Langkramer K, et al. Autologous Bone Marrow Transplantation in Patients With Subacute and Chronic Spinal Cord Injury. *Cell Transplantation*. 2006;15:675-87.

[194] Geffner LF, Santacruz P, Izurieta M, Flor L, Maldonado B, Auad AH, et al. Administration of Autologous Bone Marrow Stem Cells Into Spinal Cord Injury Patients Via Multiple Routes Is Safe and Improves Their Quality of Life: Comprehensive Case Studies. *Cell Transplantation*. 2008;17:1277-93.

[195] Yoon SH, Shim YS, Park YH, Chung JK, Nam JH, Kim MO, et al. Complete Spinal Cord Injury Treatment Using Autologous Bone Marrow Cell Transplantation and Bone Marrow Stimulation with Granulocyte Macrophage-Colony Stimulating Factor: Phase I/II Clinical Trial. *STEM CELLS*. 2007;25:2066-73.

[196] La Rocca G, Anzalone R, Corrao S, Magno F, Loria T, Lo Iacono M, et al. Isolation and characterization of Oct-4+/HLA-G+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers. *Histochemistry and Cell Biology*. 2009;131:267-82.

- [197] Troyer DL, Weiss ML. Concise Review: Wharton's Jelly-Derived Cells Are a Primitive Stromal Cell Population. *STEM CELLS*. 2008;26:591-9.
- [198] Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, et al. Human Umbilical Cord Matrix Stem Cells: Preliminary Characterization and Effect of Transplantation in a Rodent Model of Parkinson's Disease. *STEM CELLS*. 2006;24:781-92.
- [199] Yang C-C, Shih Y-H, Ko M-H, Hsu S-Y, Cheng H, Fu Y-S. Transplantation of Human Umbilical Mesenchymal Stem Cells from Wharton's Jelly after Complete Transection of the Rat Spinal Cord. *PLoS ONE*. 2008;3:e3336.
- [200] Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, or Adipose Tissue. *STEM CELLS*. 2006;24:1294-301.
- [201] Zhang H-t, Cheng H-y, Cai Y-q, Ma X, Liu W-p, Yan Z-j, et al. Comparison of adult neurospheres derived from different origins for treatment of rat spinal cord injury. *Neuroscience Letters*. 2009;458:116-21.
- [202] Doucette R. PNS-CNS transitional zone of the first cranial nerve. *The Journal of Comparative Neurology*. 1991;312:451-66.
- [203] Feron F, Perry C, Cochrane J, Licina P, Nowitzke A, Urquhart S, et al. Autologous olfactory ensheathing cell transplantation in human spinal cord injury. *Brain*. 2005;128:2951-60.
- [204] Lima C, Pratas-Vital J, Escada P, Hasse-Ferreira A, Capucho C, Peduzzi JD. Olfactory mucosa autografts in human spinal cord injury: A pilot clinical study. *Journal of Spinal Cord Medicine*. 2006;29:191-203.
- [205] Ramón-Cueto A, Cordero MI, Santos-Benito FF, Avila J. Functional Recovery of Paraplegic Rats and Motor Axon Regeneration in Their Spinal Cords by Olfactory Ensheathing Glia. *Neuron*. 2000;25:425-35.
- [206] Ramón-Cueto A, Plant GW, Avila J, Bunge MB. Long-Distance Axonal Regeneration in the Transected Adult Rat Spinal Cord Is Promoted by Olfactory Ensheathing Glia Transplants. *The Journal of Neuroscience*. 1998;18:3803-15.
- [207] Li Y, Field PM, Raisman G. Repair of Adult Rat Corticospinal Tract by Transplants of Olfactory Ensheathing Cells. *Science*. 1997;277:2000-2.

- [208] Keyvan-Fouladi N, Raisman G, Li Y. Functional Repair of the Corticospinal Tract by Delayed Transplantation of Olfactory Ensheathing Cells in Adult Rats. *The Journal of Neuroscience*. 2003;23:9428-34.
- [209] García-Álías G, López-Vales R, Forés J, Navarro X, Verdú E. Acute transplantation of olfactory ensheathing cells or Schwann cells promotes recovery after spinal cord injury in the rat. *Journal of Neuroscience Research*. 2004;75:632-41.
- [210] Nash HH, Borke RC, Anders JJ. Ensheathing Cells and Methylprednisolone Promote Axonal Regeneration and Functional Recovery in the Lesioned Adult Rat Spinal Cord. *The Journal of Neuroscience*. 2002;22:7111-20.
- [211] López-Vales R, Forés J, Navarro X, Verdú E. Chronic transplantation of olfactory ensheathing cells promotes partial recovery after complete spinal cord transection in the rat. *Glia*. 2007;55:303-11.
- [212] Guest JD, Herrera L, Margitich I, Oliveria M, Marcillo A, Casas CE. Xenografts of expanded primate olfactory ensheathing glia support transient behavioral recovery that is independent of serotonergic or corticospinal axonal regeneration in nude rats following spinal cord transection. *Experimental Neurology*. 2008;212:261-74.
- [213] Lu J, Féron F, Ho SM, Mackay-Sim A, Waite PME. Transplantation of nasal olfactory tissue promotes partial recovery in paraplegic adult rats. *Brain Research*. 2001;889:344-57.
- [214] Ramer LM, Richter MW, Roskams AJ, Tetzlaff W, Ramer MS. Peripherally-derived olfactory ensheathing cells do not promote primary afferent regeneration following dorsal root injury. *Glia*. 2004;47:189-206.
- [215] Riddell JS, Enriquez-Denton M, Toft A, Fairless R, Barnett SC. Olfactory ensheathing cell grafts have minimal influence on regeneration at the dorsal root entry zone following rhizotomy. *Glia*. 2004;47:150-67.
- [216] Steward O, Sharp K, Selvan G, Hadden A, Hofstadter M, Au E, et al. A re-assessment of the consequences of delayed transplantation of olfactory lamina propria following complete spinal cord transection in rats. *Experimental Neurology*. 2006;198:483-99.
- [217] Takami T, Oudega M, Bates ML, Wood PM, Kleitman N, Bunge MB. Schwann Cell But Not Olfactory Ensheathing Glia Transplants Improve Hindlimb Locomotor

Performance in the Moderately Contused Adult Rat Thoracic Spinal Cord. *The Journal of Neuroscience*. 2002;22:6670-81.

[218] Deng C, Gorrie C, Hayward I, Elston B, Venn M, Mackay-Sim A, et al. Survival and migration of human and rat olfactory ensheathing cells in intact and injured spinal cord. *Journal of Neuroscience Research*. 2006;83:1201-12.

[219] Boruch AV, Conners JJ, Pipitone M, Deadwyler G, Storer PD, Devries GH, et al. Neurotrophic and migratory properties of an olfactory ensheathing cell line. *Glia*. 2001;33:225-9.

[220] Li Y, Field PM, Raisman G. Regeneration of Adult Rat Corticospinal Axons Induced by Transplanted Olfactory Ensheathing Cells. *The Journal of Neuroscience*. 1998;18:10514-24.

[221] Sasaki M, Lankford KL, Zemedkun M, Kocsis JD. Identified Olfactory Ensheathing Cells Transplanted into the Transected Dorsal Funiculus Bridge the Lesion and Form Myelin. *The Journal of Neuroscience*. 2004;24:8485-93.

[222] Boyd JG, Doucette R, Kawaja MD. Defining the role of olfactory ensheathing cells in facilitating axon remyelination following damage to the spinal cord. *FASEB J*. 2005;19:694-703.

[223] Huang HY, Chen L, Wang HM, Xiu B, Li BC, Wang R, et al. Influence of patients' age on functional recovery after transplantation of olfactory ensheathing cells into injured spinal cord injury. *Chinese Medical Journal*. 2003;116:1488-91.

[224] Dobkin BH, Curt A, Guest J. Cellular Transplants in China: Observational Study from the Largest Human Experiment in Chronic Spinal Cord Injury. *Neurorehabilitation and Neural Repair*. 2006;20:5-13.

[225] Mirsky R, Jessen KR, Brennan A, Parkinson D, Dong Z, Meier C, et al. Schwann cells as regulators of nerve development. *Journal of Physiology-Paris*. 2002;96:17-24.

[226] Oudega M, Xu X-M. Schwann Cell Transplantation for Repair of the Adult Spinal Cord. *Journal of Neurotrauma*. 2006;23:453-67.

[227] Chernousov MA, Carey DJ. Schwann cell extracellular matrix molecules and their receptors. *Histology and histopathology*. 2000;15:593-601.

- [228] Duncan ID, Aguayo AJ, Bunge RP, Wood PM. Transplantation of rat schwann cells grown in tissue culture into the mouse spinal cord. *Journal of the Neurological Sciences*. 1981;49:241-52.
- [229] Bunge MB. Novel combination strategies to repair the injured mammalian spinal cord. *The journal of spinal cord medicine*. 2008;31:262-9.
- [230] Hill CE, Moon LDF, Wood PM, Bunge MB. Labeled Schwann cell transplantation: Cell loss, host Schwann cell replacement, and strategies to enhance survival. *Glia*. 2006;53:338-43.
- [231] Biernaskie J, Sparling JS, Liu J, Shannon CP, Plemel JR, Xie Y, et al. Skin-Derived Precursors Generate Myelinating Schwann Cells That Promote Remyelination and Functional Recovery after Contusion Spinal Cord Injury. *The Journal of Neuroscience*. 2007;27:9545-59.
- [232] Shields SA, Blakemore WF, Franklin RJM. Schwann cell remyelination is restricted to astrocyte-deficient areas after transplantation into demyelinated adult rat brain. *Journal of Neuroscience Research*. 2000;60:571-8.
- [233] Keyvan-Fouladi N, Raisman G, Li Y. Delayed repair of corticospinal tract lesions as an assay for the effectiveness of transplantation of Schwann cells. *Glia*. 2005;51:306-11.
- [234] Xu XM, Guénard V, Kleitman N, Aebischer P, Bunge MB. A Combination of BDNF and NT-3 Promotes Supraspinal Axonal Regeneration into Schwann Cell Grafts in Adult Rat Thoracic Spinal Cord. *Experimental Neurology*. 1995;134:261-72.
- [235] Chen A, Xu XM, Kleitman N, Bunge MB. Methylprednisolone Administration Improves Axonal Regeneration into Schwann Cell Grafts in Transected Adult Rat Thoracic Spinal Cord. *Experimental Neurology*. 1996;138:261-76.
- [236] Chau CH, Shum DKY, Li H, Pei J, Lui YY, Wirthlin L, et al. Chondroitinase ABC enhances axonal regrowth through Schwann cell-seeded guidance channels after spinal cord injury. *The FASEB Journal*. 2004;18:194-6.
- [237] Saberi H, Moshayedi P, Aghayan H-R, Arjmand B, Hosseini S-K, Emami-Razavi S-H, et al. Treatment of chronic thoracic spinal cord injury patients with autologous Schwann cell transplantation: An interim report on safety considerations and possible outcomes. *Neuroscience Letters*. 2008;443:46-50.

- [238] Halfpenny C, Benn T, Scolding N. Cell transplantation, myelin repair, and multiple sclerosis. *The Lancet Neurology*. 2002;1:31-40.
- [239] Conley BJ, Young JC, Trounson AO, Mollard R. Derivation, propagation and differentiation of human embryonic stem cells. *The International Journal of Biochemistry & Cell Biology*. 2004;36:555-67.
- [240] Hendricks WA, Pak ES, Owensby JP, Menta KJ, Glazova M, Moretto J, et al. Predifferentiated Embryonic Stem Cells Prevent Chronic Pain Behaviors and Restore Sensory Function Following Spinal Cord Injury in Mice. *ScholarOne*; 2006.
- [241] Liu S, Qu Y, Stewart TJ, Howard MJ, Chakraborty S, Holekamp TF, et al. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proceedings of the National Academy of Sciences*. 2000;97:6126-31.
- [242] McDonald JW, Liu X-Z, Qu Y, Liu S, Mickey SK, Turetsky D, et al. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med*. 1999;5:1410-2.
- [243] Nistor GI, Totoiu MO, Haque N, Carpenter MK, Keirstead HS. Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. *Glia*. 2005;49:385-96.
- [244] Keirstead HS, Nistor G, Bernal G, Totoiu M, Cloutier F, Sharp K, et al. Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cell Transplants Remyelinate and Restore Locomotion after Spinal Cord Injury. *The Journal of Neuroscience*. 2005;25:4694-705.
- [245] Li J-Y, Christophersen NS, Hall V, Soulet D, Brundin P. Critical issues of clinical human embryonic stem cell therapy for brain repair. *Trends in Neurosciences*. 2008;31:146-53.
- [246] Bretzner F, Gilbert F, Baylis F, Brownstone RM. Target Populations for First-In-Human Embryonic Stem Cell Research in Spinal Cord Injury. *Cell Stem Cell*. 2011;8:468-75.
- [247] Solbakk Jan H, Zoloth L. The Tragedy of Translation: The Case of “First Use” in Human Embryonic Stem Cell Research. *Cell Stem Cell*. 2011;8:479-81.

[248] Menlo Park, California, October 20, 2011, "Geron Presents Clinical Data Update from GRNOPC1 Spinal Cord Injury Trial", Available from: <http://ir.geron.com/phoenix.zhtml?c=67323&p=irol-newsArticle&ID=1635760&highlight=>.

[249] Menlo Park, California, November 14, 2011, "Geron to Focus on its Novel Cancer Programs", Available from URL: <http://ir.geron.com/phoenix.zhtml?c=67323&p=irol-newsArticle&ID=1635764&highlight=>.

[250] Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell*. 2006;126:663-76.

[251] Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, Chen AE, et al. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotech*. 2008;26:795-7.

[252] Kim JB, Sebastiano V, Wu G, Araújo-Bravo MJ, Sasse P, Gentile L, et al. Oct4-Induced Pluripotency in Adult Neural Stem Cells. *Cell*. 2009;136:411-9.

[253] Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, et al. Parkinson's Disease Patient-Derived Induced Pluripotent Stem Cells Free of Viral Reprogramming Factors. *Cell*. 2009;136:964-77.

[254] Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, et al. Human Induced Pluripotent Stem Cells Free of Vector and Transgene Sequences. *Science*. 2009;324:797-801.

[255] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*. 2007;131:861-72.

[256] Tsuji O, Miura K, Okada Y, Fujiyoshi K, Mukaino M, Nagoshi N, et al. Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. *Proceedings of the National Academy of Sciences*. 2010;107:12704-9.

[257] Nori S, Okada Y, Yasuda A, Tsuji O, Takahashi Y, Kobayashi Y, et al. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. *Proceedings of the National Academy of Sciences*. 2011;108:16825-30.

- [258] Bethea JR, Nagashima H, Acosta MC, Briceno C, Gomez F, Marcillo AE, et al. Systemically Administered Interleukin-10 Reduces Tumor Necrosis Factor-Alpha Production and Significantly Improves Functional Recovery Following Traumatic Spinal Cord Injury in Rats. *Journal of Neurotrauma*. 1999;16:851-63.
- [259] Plunkett JA, Yu C-G, Easton JM, Bethea JR, Yezierski RP. Effects of Interleukin-10 (IL-10) on Pain Behavior and Gene Expression Following Excitotoxic Spinal Cord Injury in the Rat. *Experimental Neurology*. 2001;168:144-54.
- [260] Zhou Z, Peng X, Insolera R, Fink DJ, Mata M. Interleukin-10 provides direct trophic support to neurons. *Journal of Neurochemistry*. 2009;110:1617-27.
- [261] Zhou Z, Peng X, Insolera R, Fink DJ, Mata M. IL-10 promotes neuronal survival following spinal cord injury. *Experimental Neurology*. 2009;220:183-90.
- [262] Takami T, Oudega M, Bethea JR, Wood PM, Kleitman N, Bunge MB. Methylprednisolone and Interleukin-10 Reduce Gray Matter Damage in the Contused Fischer Rat Thoracic Spinal Cord but Do Not Improve Functional Outcome. *Journal of Neurotrauma*. 2002;19:653-66.
- [263] Stirling DP, Koochesfahani KM, Steeves JD, Tetzlaff W. Minocycline as a Neuroprotective Agent. *The Neuroscientist*. 2005;11:308-22.
- [264] Heo K, Cho Y-J, Cho K-J, Kim H-W, Kim H-J, Shin HY, et al. Minocycline inhibits caspase-dependent and -independent cell death pathways and is neuroprotective against hippocampal damage after treatment with kainic acid in mice. *Neuroscience Letters*. 2006;398:195-200.
- [265] Festoff BW, Ameenuddin S, Arnold PM, Wong A, Santacruz KS, Citron BA. Minocycline neuroprotects, reduces microgliosis, and inhibits caspase protease expression early after spinal cord injury. *Journal of Neurochemistry*. 2006;97:1314-26.
- [266] Stirling DP, Khodarahmi K, Liu J, McPhail LT, McBride CB, Steeves JD, et al. Minocycline Treatment Reduces Delayed Oligodendrocyte Death, Attenuates Axonal Dieback, and Improves Functional Outcome after Spinal Cord Injury. *The Journal of Neuroscience*. 2004;24:2182-90.
- [267] Teng YD, Choi H, Onario RC, Zhu S, Desilets FC, Lan S, et al. Minocycline inhibits contusion-triggered mitochondrial cytochrome c release and mitigates functional

deficits after spinal cord injury. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:3071-6.

[268] Pinzon A, Marcillo A, Quintana A, Stamler S, Bunge MB, Bramlett HM, et al. A re-assessment of minocycline as a neuroprotective agent in a rat spinal cord contusion model. *Brain Research*. 2008;1243:146-51.

[269] Lee JHT, Tigchelaar S, Liu J, Stammers AMT, Streijger F, Tetzlaff W, et al. Lack of neuroprotective effects of simvastatin and minocycline in a model of cervical spinal cord injury. *Experimental Neurology*. 2010;225:219-30.

[270] Saganová K, Orendáčová J, Čížková D, Vanický I. Limited minocycline neuroprotection after balloon-compression spinal cord injury in the rat. *Neuroscience Letters*. 2008;433:246-9.

[271] Rick Hansen Institute, Vancouver, British Columbia, 2011, "Multi-Centre Clinical Research Studies", Available from: <http://www.rickhanseninstitute.org/en/information-about-sci/multi-centre-clinical-research-studies>.

[272] Schwab JM, Conrad S, Elbert T, Trautmann K, Meyermann R, Schluesener HJ. Lesional RhoA+ cell numbers are suppressed by anti-inflammatory, cyclooxygenase-inhibiting treatment following subacute spinal cord injury. *Glia*. 2004;47:377-86.

[273] Winkler T, Sharma HS, Stålberg E, Olsson Y. Indomethacin, an inhibitor of prostaglandin synthesis attenuates alteration in spinal cord evoked potentials and edema formation after trauma to the spinal cord: An experimental study in the rat. *Neuroscience*. 1993;52:1057-67.

[274] Pantovic R, Draganic P, Erakovic V, Blagovic B, Milin C, Simonic A. Effect of indomethacin on motor activity and spinal cord free fatty acid content after experimental spinal cord injury in rabbits. *Spinal cord*. 2005;43:519-26.

[275] Harada N, Taoka Y, Okajima K. Role of prostacyclin in the development of compression trauma-induced spinal cord injury in rats. *Journal of Neurotrauma*. 2006;23:1739-49.

[276] Simpson RK, Baskin DS, Dudley AW, Bogue L, Rothenberg F. The influence of long-term nifedipine or indomethacin therapy on neurologic recovery from experimental spinal cord injury. *Journal of spinal disorders*. 1991;4:420-7.

- [277] Guven MB, Cirak B, Yuceer N, Ozveren F. Is indomethacin harmful in spinal cord injury treatment? An experimental study. *Pediatric neurosurgery*. 1999;31:189-93.
- [278] Fu Q, Hue J, Li S. Nonsteroidal Anti-Inflammatory Drugs Promote Axon Regeneration via RhoA Inhibition. *The Journal of Neuroscience*. 2007;27:4154-64.
- [279] Wang X, Budel S, Baughman K, Gould G, Song K-H, Strittmatter SM. Ibuprofen Enhances Recovery from Spinal Cord Injury by Limiting Tissue Loss and Stimulating Axonal Growth. *Journal of Neurotrauma*. 2009;26:81-95.
- [280] Kwon BK, Okon E, Hillyer J, Mann C, Baptiste D, Weaver LC, et al. A Systematic Review of Non-Invasive Pharmacologic Neuroprotective Treatments for Acute Spinal Cord Injury. *Journal of Neurotrauma*. 2010;28:1545-88.
- [281] Pannu R, Barbosa E, Singh AK, Singh I. Attenuation of acute inflammatory response by atorvastatin after spinal cord injury in rats. *Journal of Neuroscience Research*. 2005;79:340-50.
- [282] Pannu R, Christie DK, Barbosa E, Singh I, Singh AK. Post-trauma Lipitor treatment prevents endothelial dysfunction, facilitates neuroprotection, and promotes locomotor recovery following spinal cord injury. *Journal of Neurochemistry*. 2007;101:182-200.
- [283] Déry M-A, Rousseau G, Benderdour M, Beaumont E. Atorvastatin prevents early apoptosis after thoracic spinal cord contusion injury and promotes locomotion recovery. *Neuroscience Letters*. 2009;453:73-6.
- [284] Mann CM, Lee JHT, Hillyer J, Stammers AMT, Tetzlaff W, Kwon BK. Lack of robust neurologic benefits with simvastatin or atorvastatin treatment after acute thoracic spinal cord contusion injury. *Experimental Neurology*. 2010;221:285-95.
- [285] Jelkmann W. Erythropoietin: structure, control of production, and function. *Physiological Reviews*. 1992;72:449-89.
- [286] Konishi Y, Chui D-H, Hirose H, Kunishita T, Tabira T. Trophic effect of erythropoietin and other hematopoietic factors on central cholinergic neurons in vitro and in vivo. *Brain Research*. 1993;609:29-35.
- [287] Gorio A, Gokmen N, Erbayraktar S, Yilmaz O, Madaschi L, Cichetti C, et al. Recombinant human erythropoietin counteracts secondary injury and markedly enhances

neurological recovery from experimental spinal cord trauma. *Proceedings of the National Academy of Sciences*. 2002;99:9450-5.

[288] Huang H, Fan S, Ji X, Zhang Y, Bao F, Zhang G. Recombinant Human Erythropoietin Protects Against Experimental Spinal Cord Trauma Injury by Regulating Expression of the Proteins MKP-1 and p-ERK. *The Journal of International Medical Research*. 2009;37:511-9.

[289] Boran BO, Colak A, Kutlay M. Erythropoietin enhances neurological recovery after experimental spinal cord injury. *Restorative neurology and neuroscience*. 2005;23:341-5.

[290] Mann C, Lee JHT, Liu J, Stammers AMT, Sohn H-M, Tetzlaff W, et al. Delayed treatment of spinal cord injury with erythropoietin or darbepoetin—A lack of neuroprotective efficacy in a contusion model of cord injury. *Experimental Neurology*. 2008;211:34-40.

[291] Pinzon A, Marcillo A, Pabon D, Bramlett HM, Bunge MB, Dietrich WD. A re-assessment of erythropoietin as a neuroprotective agent following rat spinal cord compression or contusion injury. *Experimental Neurology*. 2008;213:129-36.

[292] Faden A, Demediuk P, Panter S, Vink R. The role of excitatory amino acids and NMDA receptors in traumatic brain injury. *Science*. 1989;244:798-800.

[293] Stutzmann J-M, Pratt J, Boraud T, Gross C. The effect of riluzole on post-traumatic spinal cord injury in the rat. *NeuroReport*. 1996;7:387-92.

[294] Mu X, Azbill RD, Springer JE. Riluzole improves measures of oxidative stress following traumatic spinal cord injury. *Brain Research*. 2000;870:66-72.

[295] Springer JE, Azbill RD, Kennedy SE, George J, Geddes JW. Rapid Calpain I Activation and Cytoskeletal Protein Degradation Following Traumatic Spinal Cord Injury: Attenuation with Riluzole Pretreatment. *Journal of Neurochemistry*. 1997;69:1592-600.

[296] Kitzman PH. Effectiveness of riluzole in suppressing spasticity in the spinal cord injured rat. *Neuroscience Letters*. 2009;455:150-3.

[297] Vink R, Cernak I. Regulation of intracellular free magnesium in central nervous system injury. *Frontiers in Bioscience*. 2000;5:D656-D65.

- [298] Mendez DR, Corbett R, Macias C, Laptook A. Total and Ionized Plasma Magnesium Concentrations in Children after Traumatic Brain Injury. *Pediatric Research*. 2005;57:347-52.
- [299] Wiseman DB, Dailey AT, Lundin D, Zhou J, Lipson A, Falicov A, et al. Magnesium efficacy in a rat spinal cord injury model. *Journal of Neurosurgery: Spine*. 2009;10:308-14.
- [300] Kaptanoglu E, Beskonakli E, Solaroglu I, Kilinc A, Taskin Y. Magnesium sulfate treatment in experimental spinal cord injury: emphasis on vascular changes and early clinical results. *Neurosurgical Review*. 2003;26:283-7.
- [301] Kwon BK, Roy J, Lee JHT, Okon E, Zhang H, Marx JC, et al. Magnesium Chloride in a Polyethylene Glycol Formulation as a Neuroprotective Therapy for Acute Spinal Cord Injury: Preclinical Refinement and Optimization. *Journal of Neurotrauma*. 2009;26:1379-93.
- [302] Layden T. Kevin Everett, the road back. *Sports Illus*. 2007;107:56–67.
- [303] Inamasu J, Ichikizaki K. Mild hypothermia in neurologic emergency: An update. *Annals of Emergency Medicine*. 2002;40:220-30.
- [304] Erecinska M, Thoresen M, Silver IA. Effects of Hypothermia on Energy Metabolism in Mammalian Central Nervous System. *J Cereb Blood Flow Metab*. 2003;23:513-30.
- [305] Martinez-Arizala A, Green BA. Hypothermia in spinal cord injury. *Journal of Neurotrauma*. 1992;9 Suppl 2:S497-505.
- [306] Lo TP, Cho K-S, Garg MS, Lynch MP, Marcillo AE, Koivisto DL, et al. Systemic hypothermia improves histological and functional outcome after cervical spinal cord contusion in rats. *The Journal of Comparative Neurology*. 2009;514:433-48.
- [307] Morino T, Ogata T, Takeba J, Yamamoto H. Microglia inhibition is a target of mild hypothermic treatment after the spinal cord injury. *Spinal cord*. 2008;46:425-31.
- [308] Yu CG, Jimenez O, Marcillo AE, Weider B, Bangerter K, Dietrich WD, et al. Beneficial effects of modest systemic hypothermia on locomotor function and histopathological damage following contusion-induced spinal cord injury in rats. *Journal of Neurosurgery*. 2000;93:85-93.

- [309] Chatzipanteli K, Yanagawa Y, Marcillo AE, Kraydieh S, Yezierski RP, Dietrich WD. Posttraumatic Hypothermia Reduces Polymorphonuclear Leukocyte Accumulation Following Spinal Cord Injury in Rats. *Journal of Neurotrauma*. 2000;17:321-32.
- [310] Strain G, Waldrop R. Temperature and Vascular Volume Effects on Gastric Ulcerogenesis After Cord Transection. *Digestive Diseases and Sciences*. 2005;50:2037-42.
- [311] Levi AD, Casella G, Green BA, Dietrich WD, Vanni S, Jagid J, et al. Clinical Outcomes Using Modest Intravascular Hypothermia After Acute Cervical Spinal Cord Injury. *Neurosurgery*. 2010;66:670-7.
- [312] Levi AD, Green BA, Wang MY, Dietrich WD, Brindle T, Vanni S, et al. Clinical Application of Modest Hypothermia after Spinal Cord Injury. *Journal of Neurotrauma*. 2009;26:407-15.
- [313] Schwab M, Caroni P. Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. *The Journal of Neuroscience*. 1988;8:2381-93.
- [314] Caroni P, Schwab ME. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *The Journal of Cell Biology*. 1988;106:1281-8.
- [315] Bregman BS, Kunkel-Bagden E, Schnell L, Dai HN, Gao D, Schwab ME. Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. *Nature*. 1995;378:498-501.
- [316] Schnell L, Schwab ME. Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature*. 1990;343:269-72.
- [317] Caroni P, Schwab ME. Antibody against myelin associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron*. 1988;1:85-96.
- [318] Oudega M, Rosano C, Sadi D, Wood PM, Schwab ME, Hagg T. Neutralizing antibodies against neurite growth inhibitor ni-35/250 do not promote regeneration of sensory axons in the adult rat spinal cord. *Neuroscience*. 2000;100:873-83.

- [319] Steward O, Sharp K, Yee KM, Hofstadter M. A re-assessment of the effects of a Nogo-66 receptor antagonist on regenerative growth of axons and locomotor recovery after spinal cord injury in mice. *Experimental Neurology*. 2008;209:446-68.
- [320] Fouad K, Klusman I, Schwab ME. Regenerating corticospinal fibers in the Marmoset (*Callitrix jacchus*) after spinal cord lesion and treatment with the anti-Nogo-A antibody IN-1. *European Journal of Neuroscience*. 2004;20:2479-82.
- [321] Filbin MT. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat Rev Neurosci*. 2003;4:703-13.
- [322] Wang KC, Kim JA, Sivasankaran R, Segal R, He Z. p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature*. 2002;420:74-8.
- [323] Dickson BJ. Rho GTPases in growth cone guidance. *Current Opinion in Neurobiology*. 2001;11:103-10.
- [324] Song H-j, Poo M-m. The cell biology of neuronal navigation. *Nat Cell Biol*. 2001;3:E81-E8.
- [325] Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *The Journal of Cell Biology*. 1994;126:801-10.
- [326] Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L. Rho Signaling Pathway Targeted to Promote Spinal Cord Repair. *The Journal of Neuroscience*. 2002;22:6570-7.
- [327] Boato F, Hendrix S, Huelsenbeck SC, Hofmann F, Große G, Djalali S, et al. C3 peptide enhances recovery from spinal cord injury by improved regenerative growth of descending fiber tracts. *Journal of Cell Science*. 2010;123:1652-62.
- [328] Fournier AE, Takizawa BT, Strittmatter SM. Rho Kinase Inhibition Enhances Axonal Regeneration in the Injured CNS. *The Journal of Neuroscience*. 2003;23:1416-23.
- [329] Sung J-K, Miao L, Calvert JW, Huang L, Louis Harkey H, Zhang JH. A possible role of RhoA/Rho-kinase in experimental spinal cord injury in rat. *Brain Research*. 2003;959:29-38.

- [330] Hara M, Takasu M, Watanabe K, Noda A, Takagi T, Suzuki Y, et al. Protein kinase inhibition by fasudil hydrochloride promotes neurological recovery after spinal cord injury in rats. *Journal of Neurosurgery*. 2000;93:94-101.
- [331] Fehlings MG, Theodore N, Harrop J, Murrain G, Kuntz C, Shaffrey CI, et al. A Phase I/IIa Clinical Trial of a Recombinant Rho Protein Antagonist in Acute Spinal Cord Injury. *Journal of Neurotrauma*. 2011;28:787-96.
- [332] Zuo J, Neubauer D, Dyess K, Ferguson TA, Muir D. Degradation of Chondroitin Sulfate Proteoglycan Enhances the Neurite-Promoting Potential of Spinal Cord Tissue. *Experimental Neurology*. 1998;154:654-62.
- [333] Tester NJ, Howland DR. Chondroitinase ABC improves basic and skilled locomotion in spinal cord injured cats. *Experimental Neurology*. 2008;209:483-96.
- [334] Moon LDF, Asher RA, Rhodes KE, Fawcett JW. Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. *Nat Neurosci*. 2001;4:465-6.
- [335] Garcia-Alias G, Barkhuysen S, Buckle M, Fawcett JW. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. *Nat Neurosci*. 2009;12:1145-51.
- [336] Kim BG, Dai H-N, Lynskey JV, McAtee M, Bregman BS. Degradation of chondroitin sulfate proteoglycans potentiates transplant-mediated axonal remodeling and functional recovery after spinal cord injury in adult rats. *The Journal of Comparative Neurology*. 2006;497:182-98.
- [337] Fouad K, Schnell L, Bunge MB, Schwab ME, Liebscher T, Pearse DD. Combining Schwann Cell Bridges and Olfactory-Ensheathing Glia Grafts with Chondroitinase Promotes Locomotor Recovery after Complete Transection of the Spinal Cord. *J Neurosci*. 2005;25:1169-78.
- [338] Seikagaku Corporation, November 10, 2011, "Seikagaku announces the start of a Phase III clinical trial in Japan for SI-6603, indicated for treatment of lumbar disc herniation", Available from: <http://www.seikagaku.co.jp/english/pdf/76.pdf>.
- [339] Moreau-Fauvarque C, Kumanogoh A, Camand E, Jaillard C, Barbin G, Boquet I, et al. The Transmembrane Semaphorin Sema4D/CD100, an Inhibitor of Axonal Growth, Is

Expressed on Oligodendrocytes and Upregulated after CNS Lesion. *The Journal of Neuroscience*. 2003;23:9229-39.

[340] Benson MD, Romero MI, Lush ME, Lu QR, Henkemeyer M, Parada LF. Ephrin-B3 is a myelin-based inhibitor of neurite outgrowth. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:10694-9.

[341] Figueroa JD, Benton RL, Velazquez I, Torrado AI, Ortiz CM, Hernandez CM, et al. Inhibition of EphA7 up-regulation after spinal cord injury reduces apoptosis and promotes locomotor recovery. *Journal of Neuroscience Research*. 2006;84:1438-51.

[342] Goldshmit Y, Spanevello MD, Tajouri S, Li L, Rogers F, Pearse M, et al. EphA4 Blockers Promote Axonal Regeneration and Functional Recovery Following Spinal Cord Injury in Mice. *PLoS ONE*. 2011;6:e24636.

[343] Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME, et al. NT-3, BDNF, and NGF in the developing rat nervous system: Parallel as well as reciprocal patterns of expression. *Neuron*. 1990;5:501-9.

[344] Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H. Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. *The Journal of Cell Biology*. 1992;119:45-54.

[345] Curtis R, Adryan KM, Zhu Y, Harkness PJ, Lindsay RM, DiStefano PS. Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. *Nature*. 1993;365:253-5.

[346] Costigan M, Befort K, Karchewski L, Griffin R, D'Urso D, Allchorne A, et al. Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neuroscience*. 2002;3:16.

[347] David S, Lacroix S. Molecular Approaches to Spinal Cord Repair. *Annual Review of Neuroscience*. 2003;26:411-40.

[348] Tuszynski MH, Peterson DA, Ray J, Baird A, Nakahara Y, Gages FH. Fibroblasts Genetically Modified to Produce Nerve Growth Factor Induce Robust Neuritic Ingrowth after Grafting to the Spinal Cord. *Experimental Neurology*. 1994;126:1-14.

- [349] Grill RJ, Blesch A, Tuszynski MH. Robust Growth of Chronically Injured Spinal Cord Axons Induced by Grafts of Genetically Modified NGF-Secreting Cells. *Experimental Neurology*. 1997;148:444-52.
- [350] Tuszynski MH, Gabriel K, Gage FH, Suhr S, Meyer S, Rosetti A. Nerve Growth Factor Delivery by Gene Transfer Induces Differential Outgrowth of Sensory, Motor, and Noradrenergic Neurites after Adult Spinal Cord Injury. *Experimental Neurology*. 1996;137:157-73.
- [351] Kwon BK, Liu J, Messerer C, Kobayashi NR, McGraw J, Oschipok L, et al. Survival and regeneration of rubrospinal neurons 1 year after spinal cord injury. *Proceedings of the National Academy of Sciences*. 2002;99:3246-51.
- [352] Liu Y, Kim D, Himes BT, Chow SY, Schallert T, Murray M, et al. Transplants of Fibroblasts Genetically Modified to Express BDNF Promote Regeneration of Adult Rat Rubrospinal Axons and Recovery of Forelimb Function. *The Journal of Neuroscience*. 1999;19:4370-87.
- [353] Bregman BS, McAtee M, Dai HN, Kuhn PL. Neurotrophic Factors Increase Axonal Growth after Spinal Cord Injury and Transplantation in the Adult Rat. *Experimental Neurology*. 1997;148:475-94.
- [354] Bradbury EJ, Khemani S, Von R, King, Priestley JV, McMahon SB. NT-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. *European Journal of Neuroscience*. 1999;11:3873-83.
- [355] Tuszynski MH, Grill R, Jones LL, Brant A, Blesch A, Löw K, et al. NT-3 gene delivery elicits growth of chronically injured corticospinal axons and modestly improves functional deficits after chronic scar resection. *Experimental Neurology*. 2003;181:47-56.
- [356] Miller FD, Kaplan DR. Neurotrophin signalling pathways regulating neuronal apoptosis. *Cellular and molecular life sciences : CMLS*. 2001;58:1045-53.
- [357] Blesch A, Tuszynski MH. Cellular GDNF delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination. *The Journal of Comparative Neurology*. 2003;467:403-17.
- [358] Facchiano F, Fernandez E, Mancarella S, Maira G, Miscusi M, D'Arcangelo D, et al. Promotion of regeneration of corticospinal tract axons in rats with recombinant

vascular endothelial growth factor alone and combined with adenovirus coding for this factor. *Journal of Neurosurgery*. 2002;97:161-8.

[359] Kasai M, Jikoh T, Fukumitsu H, Furukawa S. FGF-2-responsive and spinal cord-resident cells improve locomotor function after spinal cord injury. *Journal of Neurotrauma*. 2010.

[360] Kwon BK, Liu J, Oschipok L, Teh J, Liu ZW, Tetzlaff W. Rubrospinal neurons fail to respond to brain-derived neurotrophic factor applied to the spinal cord injury site 2 months after cervical axotomy. *Experimental Neurology*. 2004;189:45-57.

[361] Stuart C A. Nerve growth factor for the treatment of diabetic neuropathy: What went wrong, what went right, and what does the future hold? In: David T, editor. *International Review of Neurobiology*: Academic Press; 2002. p. 393-413.

[362] Tuszynski MH, Thal L, Pay M, Salmon DP, U HS, Bakay R, et al. A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nat Med*. 2005;11:551-5.

[363] Wu J-C, Huang W-C, Tsai Y-A, Chen Y-C, Cheng H. Nerve repair using acidic fibroblast growth factor in human cervical spinal cord injury: a preliminary Phase I clinical study. *Journal of Neurosurgery: Spine*. 2008;8:208-14.

[364] Spencer T, Filbin MT. A role for cAMP in regeneration of the adult mammalian CNS. *Journal of Anatomy*. 2004;204:49-55.

[365] Neumann S, Bradke F, Tessier-Lavigne M, Basbaum AI. Regeneration of Sensory Axons within the Injured Spinal Cord Induced by Intraganglionic cAMP Elevation. *Neuron*. 2002;34:885-93.

[366] Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, Bregman BS, et al. Spinal Axon Regeneration Induced by Elevation of Cyclic AMP. *Neuron*. 2002;34:895-903.

[367] Kolb A, Busby S, Buc II, Garges S, Adhya S. Transcriptional Regulation by cAMP and its Receptor Protein. *Annual Review of Biochemistry*. 1993;62:749-97.

[368] Schwartz JH. The many dimensions of cAMP signaling. *Proceedings of the National Academy of Sciences*. 2001;98:13482-4.

[369] Fouad K, Ghosh M, Vavrek R, Tse AD, Pearse DD. Dose and Chemical Modification Considerations for Continuous Cyclic AMP Analog Delivery to the Injured CNS. *Journal of Neurotrauma*. 2009;26:733-40.

- [370] Kajana S, Goshgarian HG. Systemic administration of rolipram increases medullary and spinal cAMP and activates a latent respiratory motor pathway after high cervical spinal cord injury. *The journal of spinal cord medicine*. 2009;32:175-82.
- [371] Whitaker CM, Beaumont E, Wells MJ, Magnuson DSK, Hetman M, Onifer SM. Rolipram attenuates acute oligodendrocyte death in the adult rat ventrolateral funiculus following contusive cervical spinal cord injury. *Neuroscience Letters*. 2008;438:200-4.
- [372] Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, et al. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. *Nat Med*. 2004;10:610-6.
- [373] Bretzner F, Plemel JR, Liu J, Richter M, Roskams AJ, Tetzlaff W. Combination of olfactory ensheathing cells with local versus systemic cAMP treatment after a cervical rubrospinal tract injury. *Journal of Neuroscience Research*. 2010;88:2833-46.
- [374] Nout YS, Culp E, Schmidt MH, Tovar CA, Pröschel C, Mayer-Pröschel M, et al. Glial restricted precursor cell transplant with cyclic adenosine monophosphate improved some autonomic functions but resulted in a reduced graft size after spinal cord contusion injury in rats. *Experimental Neurology*. 2011;227:159-71.
- [375] Alilain WJ, Horn KP, Hu H, Dick TE, Silver J. Functional regeneration of respiratory pathways after spinal cord injury. *Nature*. 2011;475:196-200.
- [376] Tom VJ, Sandrow-Feinberg HR, Miller K, Santi L, Connors T, Lemay MA, et al. Combining Peripheral Nerve Grafts and Chondroitinase Promotes Functional Axonal Regeneration in the Chronically Injured Spinal Cord. *The Journal of Neuroscience*. 2009;29:14881-90.
- [377] Houle JD, Tom VJ, Mayes D, Wagoner G, Phillips N, Silver J. Combining an Autologous Peripheral Nervous System "Bridge" and Matrix Modification by Chondroitinase Allows Robust, Functional Regeneration beyond a Hemisection Lesion of the Adult Rat Spinal Cord. *The Journal of Neuroscience*. 2006;26:7405-15.
- [378] Yoon SH, Shim YS, Park YH, Chung JK, Nam JH, Kim MO, et al. Complete spinal cord injury treatment using autologous bone marrow cell transplantation and bone marrow stimulation with granulocyte macrophage-colony stimulating factor: Phase I/II clinical trial. *Stem cells (Dayton, Ohio)*. 2007;25:2066-73.

- [379] Ziv Y, Avidan H, Pluchino S, Martino G, Schwartz M. Synergy between immune cells and adult neural stem/progenitor cells promotes functional recovery from spinal cord injury. *Proceedings of the National Academy of Sciences*. 2006;103:13174-9.
- [380] Meng X-t, Li C, Dong Z-y, Liu J-m, Li W, Liu Y, et al. Co-transplantation of bFGF-expressing amniotic epithelial cells and neural stem cells promotes functional recovery in spinal cord-injured rats. *Cell Biology International*. 2008;32:1546-58.
- [381] Ishii K, Nakamura M, Dai H, Finn TP, Okano H, Toyama Y, et al. Neutralization of ciliary neurotrophic factor reduces astrocyte production from transplanted neural stem cells and promotes regeneration of corticospinal tract fibers in spinal cord injury. *Journal of Neuroscience Research*. 2006;84:1669-81.
- [382] Kim KN, Oh SH, Lee KH, Yoon DH. Effect of human mesenchymal stem cell transplantation combined with growth factor infusion in the repair of injured spinal cord. In: Chang JW, Katayama Y, Yamamoto T, editors. *Advances in Functional and Reparative Neurosurgery*: Springer Vienna; 2006. p. 133-6.
- [383] Lu P, Jones LL, Tuszynski MH. BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. *Experimental Neurology*. 2005;191:344-60.
- [384] Lu P, Yang H, Jones LL, Filbin MT, Tuszynski MH. Combinatorial Therapy with Neurotrophins and cAMP Promotes Axonal Regeneration beyond Sites of Spinal Cord Injury. *The Journal of Neuroscience*. 2004;24:6402-9.
- [385] Chiba Y, Kuroda S, Shichinohe H, Hokari M, Osanai T, Maruichi K, et al. Synergistic effects of bone marrow stromal cells and a Rho kinase (ROCK) inhibitor, Fasudil on axon regeneration in rat spinal cord injury. *Neuropathology*. 2010;30:241-50.
- [386] Fang K-M, Chen J-K, Hung S-C, Chen M-C, Wu Y-T, Wu T-J, et al. Effects of Combinatorial Treatment with Pituitary Adenylate Cyclase Activating Peptide and Human Mesenchymal Stem Cells on Spinal Cord Tissue Repair. *PLoS ONE*. 2010;5:e15299.
- [387] Ruitenberg MJ, Levison DB, Lee SV, Verhaagen J, Harvey AR, Plant GW. NT-3 expression from engineered olfactory ensheathing glia promotes spinal sparing and regeneration. *Brain*. 2005;128:839-53.

- [388] Pearse DD, Sanchez AR, Pereira FC, Andrade CM, Puzis R, Pressman Y, et al. Transplantation of Schwann cells and/or olfactory ensheathing glia into the contused spinal cord: Survival, migration, axon association, and functional recovery. *Glia*. 2007;55:976-1000.
- [389] Cao L, Liu L, Chen ZY, Wang LM, Ye JL, Qiu HY, et al. Olfactory ensheathing cells genetically modified to secrete GDNF to promote spinal cord repair. *Brain*. 2004;127:535-49.
- [390] Menei P, Montero-Menei C, Whitemore SR, Bunge RP, Bunge MB. Schwann cells genetically modified to secrete human BDNF promote enhanced axonal regrowth across transected adult rat spinal cord. *European Journal of Neuroscience*. 1998;10:607-21.
- [391] Guest JD, Hesse D, Schnell L, Schwab ME, Bunge MB, Bunge RP. Influence of IN-1 antibody and acidic FGF-fibrin glue on the response of injured corticospinal tract axons to human Schwann cell grafts. *Journal of Neuroscience Research*. 1997;50:888-905.
- [392] Nikulina E, Tidwell JL, Dai HN, Bregman BS, Filbin MT. The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:8786-90.
- [393] Kimura H, Yoshikawa M, Matsuda R, Toriumi H, Nishimura F, Hirabayashi H, et al. Transplantation of embryonic stem cell-derived neural stem cells for spinal cord injury in adult mice. *Neurological Research*. 2005;27:812-9.
- [394] Cao Q, He Q, Wang Y, Cheng X, Howard RM, Zhang Y, et al. Transplantation of Ciliary Neurotrophic Factor-Expressing Adult Oligodendrocyte Precursor Cells Promotes Remyelination and Functional Recovery after Spinal Cord Injury. *The Journal of Neuroscience*. 2010;30:2989-3001.
- [395] Yick L-W, So K-F, Cheung P-T, Wu W-T. Lithium Chloride Reinforces the Regeneration-Promoting Effect of Chondroitinase ABC on Rubrospinal Neurons after Spinal Cord Injury. *Journal of Neurotrauma*. 2004;21:932-43.
- [396] García-Alías G, Petrosyan HA, Schnell L, Horner PJ, Bowers WJ, Mendell LM, et al. Chondroitinase ABC Combined with Neurotrophin NT-3 Secretion and NR2D

Expression Promotes Axonal Plasticity and Functional Recovery in Rats with Lateral Hemisection of the Spinal Cord. *The Journal of Neuroscience*. 2011;31:17788-99.

[397] Caggiano AO, Zimmer MP, Ganguly A, Blight AR, Gruskin EA. Chondroitinase ABCI Improves Locomotion and Bladder Function following Contusion Injury of the Rat Spinal Cord. *Journal of Neurotrauma*. 2005;22:226-39.

[398] Nothias J-M, Mitsui T, Shumsky JS, Fischer I, Antonacci MD, Murray M. Combined Effects of Neurotrophin Secreting Transplants, Exercise, and Serotonergic Drug Challenge Improve Function in Spinal Rats. *Neurorehabilitation and Neural Repair*. 2005;19:296-312.

[399] Klapka N, Hermanns S, Straten G, Masannek C, Duis S, Hamers FPT, et al. Suppression of fibrous scarring in spinal cord injury of rat promotes long-distance regeneration of corticospinal tract axons, rescue of primary motoneurons in somatosensory cortex and significant functional recovery. *European Journal of Neuroscience*. 2005;22:3047-58.

[400] Ditor DS, John SM, Roy J, Marx JC, Kittmer C, Weaver LC. Effects of polyethylene glycol and magnesium sulfate administration on clinically relevant neurological outcomes after spinal cord injury in the rat. *Journal of Neuroscience Research*. 2007;85:1458-67.

[401] Salgado AJ, Coutinho OP, Reis RL. Bone Tissue Engineering: State of the Art and Future Trends. *Macromolecular Bioscience*. 2004;4:743-65.

[402] Nomura H, Tator CH, Shoichet MS. Bioengineered Strategies for Spinal Cord Repair. *Journal of Neurotrauma*. 2006;23:496-507.

[403] Zimmermann W-H, Schneiderbanger K, Schubert P, Didié M, Münzel F, Heubach JF, et al. Tissue Engineering of a Differentiated Cardiac Muscle Construct. *Circulation Research*. 2002;90:223-30.

[404] Oliveira JT, Reis RL. Polysaccharide-based materials for cartilage tissue engineering applications. *Journal of Tissue Engineering and Regenerative Medicine*. 2011;5:421-36.

[405] Atala A. Engineering tissues, organs and cells. *Journal of Tissue Engineering and Regenerative Medicine*. 2007;1:83-96.

- [406] Jukes JM, van Blitterswijk CA, de Boer J. Skeletal tissue engineering using embryonic stem cells. *Journal of Tissue Engineering and Regenerative Medicine*. 2010;4:165-80.
- [407] Langer R, Vacanti JP. Tissue engineering. *Science*. 1993;260:920-6.
- [408] Kneser U, Schaefer DJ, Munder B, Klemm C, Andree C, Stark GB. Tissue engineering of bone. *Minimally Invasive Therapy & Allied Technologies*. 2002;11:107-16.
- [409] Dietmar W H. Scaffolds in tissue engineering bone and cartilage. *Biomaterials*. 2000;21:2529-43.
- [410] Silva NA, Sousa RA, Pires AO, Sousa N, Salgado AJ, Reis RL. Interactions between Schwann and olfactory ensheathing cells with a starch/polycaprolactone scaffold aimed at spinal cord injury repair. *Journal of Biomedical Materials Research Part A*. 2012;100A:470-6.
- [411] Silva GA, Ducheyne P, Reis RL. Materials in particulate form for tissue engineering. 1. Basic concepts. *Journal of Tissue Engineering and Regenerative Medicine*. 2007;1:4-24.
- [412] Stokols S, Tuszynski MH. The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. *Biomaterials*. 2004;25:5839-46.
- [413] Nomura H, Zahir T, Kim H, Katayama Y, Kulbatski I, Morshead CM, et al. Extramedullary Chitosan Channels Promote Survival of Transplanted Neural Stem and Progenitor Cells and Create a Tissue Bridge After Complete Spinal Cord Transection. *Tissue Engineering Part A*. 2008;14:649-65.
- [414] Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Pedro AJ, Leite-Almeida H, et al. Development and Characterization of a Novel Hybrid Tissue Engineering Based Scaffold for Spinal Cord Injury Repair. *Tissue Engineering Part A*. 2010;16:45-54.
- [415] Wang M, Zhai P, Chen X, Schreyer DJ, Sun X, Cui F. Bioengineered Scaffolds for Spinal Cord Repair. *Tissue Engineering Part B: Reviews*. 2011;17:177-94.
- [416] Perale G, Rossi F, Sundstrom E, Bacchiega S, Masi M, Forloni G, et al. Hydrogels in Spinal Cord Injury Repair Strategies. *ACS Chemical Neuroscience*. 2011;2:336-45.

- [417] Nisbet DR, Crompton KE, Horne MK, Finkelstein DI, Forsythe JS. Neural tissue engineering of the CNS using hydrogels. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. 2008;87B:251-63.
- [418] Hejcl A, Lesny P, Pradny M, Michalek J, Jendelova P, Stulik J, et al. Biocompatible Hydrogels in Spinal Cord Injury Repair. *Physiological Research*. 2008;57:S121-S32.
- [419] Silva N, Cooke M, Tam R, Sousa N, Salgado AJ, Reis RL, et al. The Effects of Peptide Modified Gellan Gum and Olfactory Ensheathing Glia Cells on Neural Stem/Progenitor Cell Fate. *Biomaterials*. 2012;accepted for publication.
- [420] Ribeiro-Samy S, Silva N, Correlo VM, Silva R, Fraga JS, Pinto L, et al. Development and Characterization of a PHB-HV-based 3D Scaffold for a Tissue Engineering and Cell-Therapy combinatorial approach for Spinal Cord Injury Regeneration, Submitted.
- [421] Friedman JA, Windebank AJ, Moore MJ, Spinner RJ, Currier BL, Yaszemski MJ. Biodegradable Polymer Grafts for Surgical Repair of the Injured Spinal Cord. *Neurosurgery*. 2002;51:742-52.
- [422] Li W-J, Laurencin CT, Caterson EJ, Tuan RS, Ko FK. Electrospun nanofibrous structure: A novel scaffold for tissue engineering. *Journal of Biomedical Materials Research*. 2002;60:613-21.
- [423] Xie J, Willerth SM, Li X, Macewan MR, Rader A, Sakiyama-Elbert SE, et al. The differentiation of embryonic stem cells seeded on electrospun nanofibers into neural lineages. *Biomaterials*. 2009;30:354-62.
- [424] Doshi J, Reneker DH. Electrospinning process and applications of electrospun fibers. *Journal of Electrostatics*. 1995;35:151-60.
- [425] Hejcl A, Šedý J, Kapcalová M, Toro DA, Amemori T, Lesný P, et al. HPMa-RGD Hydrogels Seeded with Mesenchymal Stem Cells Improve Functional Outcome in Chronic Spinal Cord Injury. *Stem Cells and Development*. 2010;19:1535-46.
- [426] Reynolds LF, Bren MC, Wilson BC, Gibson GD, Shoichet MS, Murphy RJL. Transplantation of porous tubes following spinal cord transection improves hindlimb function in the rat. *Spinal cord*. 2007;46:58-64.

- [427] Tsai EC, Dalton PD, Shoichet MS, Tator CH. Matrix inclusion within synthetic hydrogel guidance channels improves specific supraspinal and local axonal regeneration after complete spinal cord transection. *Biomaterials*. 2006;27:519-33.
- [428] Nomura H, Katayama YM, Shoichet MS, Tator CH. Complete Spinal Cord Transection Treated by Implantation of A Reinforced Synthetic Hydrogel Channel Results in Syringomyelia and Caudal Migration of the Rostral Stump. *Neurosurgery*. 2006;59:183-92.
- [429] Shi R, Borgens RB. Acute Repair of Crushed Guinea Pig Spinal Cord by Polyethylene Glycol. *Journal of Neurophysiology*. 1999;81:2406-14.
- [430] Luo J, Borgens R, Shi R. Polyethylene glycol immediately repairs neuronal membranes and inhibits free radical production after acute spinal cord injury. *Journal of Neurochemistry*. 2002;83:471-80.
- [431] Luo J, Shi R. Diffusive oxidative stress following acute spinal cord injury in guinea pigs and its inhibition by polyethylene glycol. *Neuroscience Letters*. 2004;359:167-70.
- [432] Piantino J, Burdick JA, Goldberg D, Langer R, Benowitz LI. An injectable, biodegradable hydrogel for trophic factor delivery enhances axonal rewiring and improves performance after spinal cord injury. *Experimental Neurology*. 2006;201:359-67.
- [433] Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, et al. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. *Proceedings of the National Academy of Sciences*. 2002;99:3024-9.
- [434] Tysseling-Mattiace VM, Sahni V, Niece KL, Birch D, Czeisler C, Fehlings MG, et al. Self-Assembling Nanofibers Inhibit Glial Scar Formation and Promote Axon Elongation after Spinal Cord Injury. *The Journal of Neuroscience*. 2008;28:3814-23.
- [435] Petter-Puchner AH, Froetscher W, Krametter-Froetscher R, Lorinson D, Redl H, van Griensven M. The long-term neurocompatibility of human fibrin sealant and equine collagen as biomatrices in experimental spinal cord injury. *Experimental and Toxicologic Pathology*. 2007;58:237-45.
- [436] Houweling DA, Lankhorst AJ, Gispen WH, Bär PR, Joosten EAJ. Collagen Containing Neurotrophin-3 (NT-3) Attracts Regrowing Injured Corticospinal Axons in

the Adult Rat Spinal Cord and Promotes Partial Functional Recovery. *Experimental Neurology*. 1998;153:49-59.

[437] Li X, Yang Z, Zhang A, Wang T, Chen W. Repair of thoracic spinal cord injury by chitosan tube implantation in adult rats. *Biomaterials*. 2009;30:1121-32.

[438] Kleinman HK, Martin GR. Matrigel: Basement membrane matrix with biological activity. *Seminars in Cancer Biology*. 2005;15:378-86.

[439] Lee S-H, Chung Y-N, Kim Y-H, Kim Y-J, Park J-P, Kwon D-K, et al. Effects of human neural stem cell transplantation in canine spinal cord hemisection. *Neurological Research*. 2009;31:996-1002.

[440] Patel V, Joseph G, Patel A, Patel S, Bustin D, Mawson D, et al. Suspension Matrices for Improved Schwann-Cell Survival after Implantation into the Injured Rat Spinal Cord. *Journal of Neurotrauma*. 2010;27:789-801.

[441] Samadikuchaksaraei A. An overview of tissue engineering approaches for management of spinal cord injuries. *Journal of NeuroEngineering and Rehabilitation*. 2007;4:15.

[442] Taylor SJ, McDonald Iii JW, Sakiyama-Elbert SE. Controlled release of neurotrophin-3 from fibrin gels for spinal cord injury. *Journal of Controlled Release*. 2004;98:281-94.

[443] Kim H, Zahir T, Tator CH, Shoichet MS. Effects of Dibutyryl Cyclic-AMP on Survival and Neuronal Differentiation of Neural Stem/Progenitor Cells Transplanted into Spinal Cord Injured Rats. *PLoS ONE*. 2011;6:e21744.

[444] Pritchard CD, Slotkin JR, Yu D, Dai H, Lawrence MS, Bronson RT, et al. Establishing a model spinal cord injury in the African green monkey for the preclinical evaluation of biodegradable polymer scaffolds seeded with human neural stem cells. *Journal of Neuroscience Methods*. 2010;188:258-69.

[445] Cheng H, Cao Y, Olson L. Spinal Cord Repair in Adult Paraplegic Rats: Partial Restoration of Hind Limb Function. *Science*. 1996;273:510-3.

[446] Kim Y-t, Caldwell J-M, Bellamkonda RV. Nanoparticle-mediated local delivery of methylprednisolone after spinal cord injury. *Biomaterials*. 2009;30:2582-90.

[447] Tobias CA, Han SSW, Shumsky JS, Kim D, Tumolo M, Dhoot NO, et al. Alginate Encapsulated BDNF-Producing Fibroblast Grafts Permit Recovery of Function after

Spinal Cord Injury in the Absence of Immune Suppression. *Journal of Neurotrauma*. 2005;22:138-56.

[448] Gilbert RJ, Rivet CJ, Zuidema JM, Popovich PG. Biomaterial Design Considerations for Repairing the Injured Spinal Cord. *Crit Rev Biomed Eng*. 2011;39:125-80.

[449] Fawcett JW, Curt A, Steeves JD, Coleman WP, Tuszynski MH, Lammertse D, et al. Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP panel: spontaneous recovery after spinal cord injury and statistical power needed for therapeutic clinical trials. *Spinal cord*. 2006;45:190-205.

[450] Steeves JD, Lammertse D, Curt A, Fawcett JW, Tuszynski MH, Ditunno JF, et al. Guidelines for the conduct of clinical trials for spinal cord injury (SCI) as developed by the ICCP panel: clinical trial outcome measures. *Spinal cord*. 2006;45:206-21.

[451] Tuszynski MH, Steeves JD, Fawcett JW, Lammertse D, Kalichman M, Rask C, et al. Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP Panel: clinical trial inclusion/exclusion criteria and ethics. *Spinal cord*. 2006;45:222-31.

[452] Lammertse D, Tuszynski MH, Steeves JD, Curt A, Fawcett JW, Rask C, et al. Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP panel: clinical trial design. *Spinal cord*. 2006;45:232-42.

SECTION II

CHAPTER II

Materials & Methods

CHAPTER II

Materials & Methods

This chapter describes the materials and experimental methods used in the scope of this thesis. Although each part of the work is accompanied by its specific materials and methods section, this chapter intends to condensate and compile the relevant information on this matter. This exercise is expected to provide the reader a more comprehensive view of experimental and analytical tools used, as well as to facilitate their use by others.

1. Materials

1.1 Starch Polycaprolactone (SPCL)

A blend material consisting of 30/70% (wt) corn starch and polycaprolactone was used to produce tubular porous scaffolds. The objective of combining these materials is the improved performance they might confer ultimately to the tissue engineered construct in an *in vivo* scenario. Starch is a natural polymer made of a combination of two polymeric carbohydrates, amylose and amylopectin [1, 2]. Amylose is a linear polymer of several thousand glucose residues $\alpha(1\rightarrow4)$ -linked, while amylopectin consists of $\alpha(1\rightarrow4)$ -linked glucose residues with $\alpha(1\rightarrow6)$ branch points (Fig. 1). Starch is a renewable and inherently biodegradable polymer that has been put forward as a cell support material in combination with synthetic polymers such as polycaprolactone (PCL), polylactic acid (PLA), ethyl vinyl alcohol (EVOH), and cellulose acetate (CA). Several studies have been conducted with these materials, mainly in bone tissue engineering [3-6].

Polycaprolactone (Fig. 1a) is a semicrystalline biodegradable polymer belonging to the family of poly- α -hydroxyl polyesters. PCL has a low melting point of around 60°C, low viscosity, and it is easy to process [7]. PCL has been previously approved by FDA for medical applications and has also been extensively studied for tissue engineering applications [8, 9]. The SPCL blend used was obtained from Novamont S.p.A., Italy.

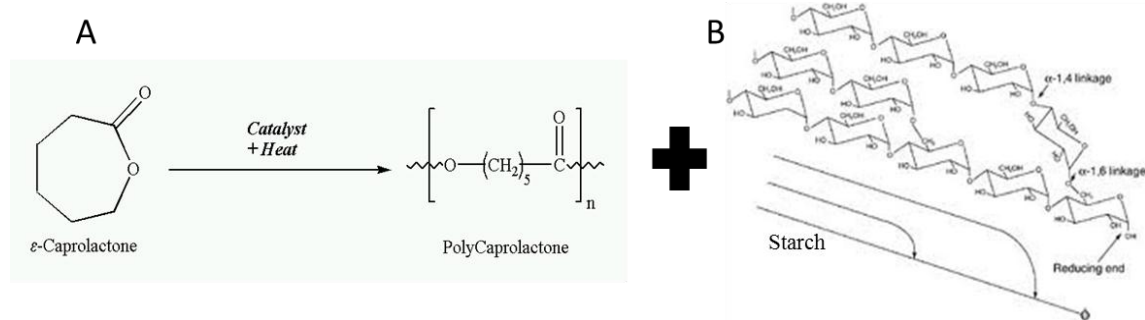


Figure II.1. The polymeric form of caprolactone (A) is added to corn starch (B) to form the SCPL polymer.

1.2. Gellan Gum

Gellan Gum (GG) is a linear anionic polysaccharide produced by bacterial fermentation and its basic structural unit is composed of glucose, rhamnose and glucuronic acid (Figure 2). This material was initially described by Moorthouse and colleagues in 1981 [10] and has a broad use in the food industry and biomedical fields, mostly due to its processing into transparent gels that are resistant to heat and acid stress [11]. It is also FDA approved since 1992 [12]. Gellan Gum has an ionotropic gelation strongly influenced by the chemical nature and quantity of cations present in the solution. Divalent cations promote a stronger gelation than monovalent cations [11]. In tissue engineering, this

material has been suggested mostly for cartilage regeneration [13, 14]. The Gellan Gum was purchased from Sigma-Aldrich, St. Louis, USA.

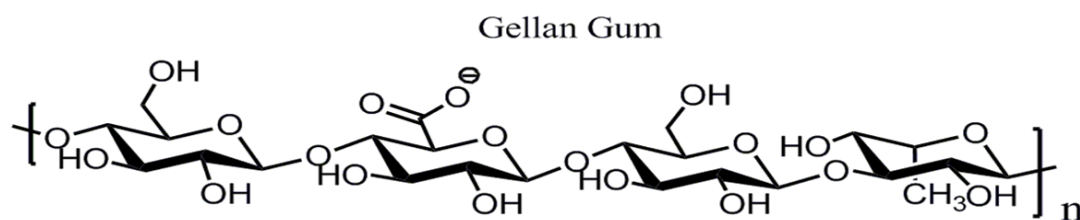


Figure II.2. Chemical structure of Gellan Gum. This polymer is composed of repeated tetrasaccharide units of glucose, glucuronic acid, and rhamnose residues in a 2:1:1 ratio

2. Scaffolds Production

2.1 Starch Polycaprolactone Scaffolds

Tubular SPCL scaffolds were developed by rapid prototyping techniques. Rapid prototyping is a computerized fabrication technique that can rapidly produce a highly complex three-dimensional physical objects using data generated by computer assisted design (CAD) systems, computer-based medical imaging modalities, digitizers and other data makers [15]. Rapid prototyping uses the underlying concept of layered manufacturing and whereby each part of the 3D objects is constructed in a layer-by-layer manner [16].

The SPCL tubular scaffolds were processed in a two-step methodological approach:

- 1) Porous sheets featuring different pore geometries were produced by 3D plotting rapid prototyping (Bioplotter®; Envisiontec GmbH, Marl, Germany), using two different inter filament orientations (90° and 45°);

2) Tubular scaffolds were obtained by rolling up porous sheets around a cylinder and subsequent heat treatment at 65° C during 30 min for inducing the adhesion between filaments.

Up to six different porous tubular scaffold designs were obtained featuring a single or double layer combined with three different filaments' oriented relative to the tube main axis: filaments oriented at 0° and 90° relative to the tube main axis (90/1-2, further referred as 90/1 and 90/2 for structures with one or two layers), filaments oriented at 0° and 45° relative to the tube main axis (45/1-2, further referred as 45/1 and 45/2), and filaments oriented at 45° relative to the tube main axis (X/1-2, further referred as X/1 and X/2). As example of the adopted scaffold references, processing combination 90/2 refers to a double layer scaffold featuring filaments' orientated at 0° and 90° relative to the tube main axis, while 45/1 refers to a single layer scaffold featuring filaments' orientated at 45° relative to the tube main axis.

2.2 Gellan Gum Hydrogels

Gellan gum powder was mixed with distilled water under constant stirring at room temperature. The solution was progressively heated to 90°C, under which complete and homogenous dispersion of the material was obtained. The solution was kept at this temperature during 20 minutes. Afterwards, CaCl₂ (Merck, DE) was added to obtain a final concentration of 0.03% (w/v) in the gellan gum solution and the temperature was progressively decreased to 50°C. The gellan gum was then injected in the desired structures and allowed to rest at room temperature for 3 minutes in order to form a solid gel. With the purpose of obtain the hybrid scaffold (Chapter III), gellan gum was used in a 1% (w/v) concentration. In chapter VI gellan gum concentration used was 0.5%

(w/v). Moreover, in that chapter gellan gum was not heated until 90°C. Instead, and in order to protect the GRGDS sequence, gellan gum powder was dissolved by overnight incubation in distilled water.

2.3 Three-Dimensional Hybrid Structures

The integration of gellan gum hydrogel into the central canal of the SPCL tubular scaffolds was performed by placing the tubular scaffolds into a specially designed mold (fig. 3), and by filling the empty volume with a gellan gum hydrogel obtained as described above.

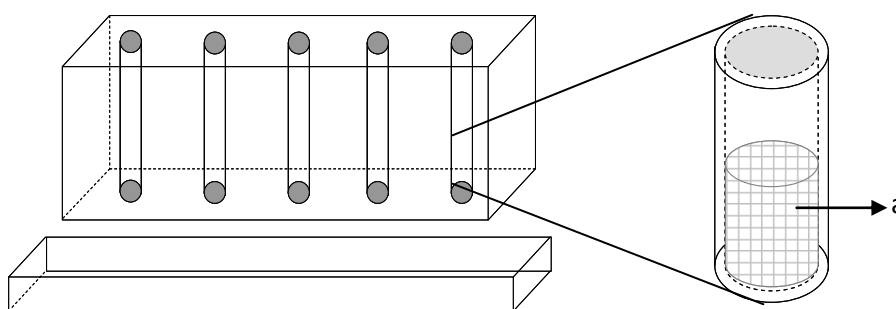


Figure II.3. Schematic representation of the mold used to combine the SPCL and the gellan gum hydrogel. (a) SPCL scaffolds.

3. Gellan Gum Functionalization by Click Chemistry

Click chemistry was introduced by Barry Sharpless in 2001 and describes chemistry tailored to generate substances quickly and reliably by joining small units together [17]. The reactions in click chemistry must be wide in scope, give very high chemical yields, generate only inoffensive byproducts, and be physiologically stable. Some of these click chemistry reactions, included the one used in the scope of this thesis, are based on the

novel principles introduced by the Otto Diels and Kurt Alder. The Diels-Alder reaction is an organic chemical reaction between a conjugated diene and an alkene to form a substituted cyclohexene [18]. These principles were applied in the work described in chapter VI aiming the immobilization of a bioactive peptide in the gellan gum hydrogel. In order to do so, 3 separated protocols had to be performed (Fig. 4). First the gellan gum was modified with a furan group, then a maleimide-modified GRDGS sequence had to be synthesized, and finally the furan and maleimide were combined in order to create a GRGDS-modified gellan gum.

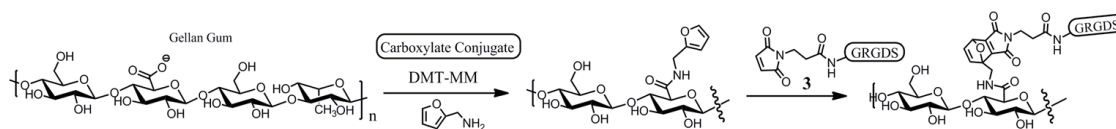


Figure II.4. Immobilization of the GRGDS peptide in the gellan gum hydrogel by Diels-Alder click chemistry.

3.1 Synthesis of Furan-Modified Gellan Gum Hydrogel

Gellan gum (Sigma, USA) was first dissolved in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (100 mM, pH 5.5) at 37 °C. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Sigma, USA) and furfurylamine (Furan, Acros Organics, Belgium) were then added in a 4:1 molar ratio (relative to the –COOH groups in gellan gum) and stirred at 37 °C for 48 hours. The solution was then dialyzed (M_w cutoff 12-14kDaltons, Spectrum Labs, USA) alternately against distilled water and PBS (0.1M and pH 7.2) for 5 days. Finally, water was removed by lyophilization to obtain furan-modified gellan gum (furan-GG) as a white powder. $^1\text{H-NMR}$ spectra were used to analyze the degree of furan substitution. $^1\text{H-NMR}$ was recorded in D_2O on a Varian Mercury-400 MHz NMR spectrometer (Palo Alto, USA). As a negative control, GG was incubated with furan in the absence of DMTMM.

3.2 Synthesis of Maleimide-Modified GRGDS Peptide

Maleimide-modified GRGDS (mal-GRGDS) peptide was prepared by linear solid-phase synthesis (SPS) using standard Fmoc chemistry [19]. Fmoc-serine Wang resin (Anaspect, USA) with a loading capacity of 0.48 mmol/g was swollen in DMF (Sigma, USA) for 30 minutes. The solution was then drained and 20% of piperidine (Caledon, Canada) in DMF was added. After 30 minutes of incubation the resin was washed with DMF and tested for free amines using 1 % trinitrobenzene sulfonate, TNBS (TCI America, USA), in DMF. The first building block (Fmoc-asp-OH, 3.0 equivalents, all amino acids are from NovaBiochem, Germany) was pre-mixed with 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate, HCTU (3.0 equivalents, Anaspect, USA) in DMF for 15 min, then transferred to the SPS flask. Diisopropylethylamine, DIPEA (4.0 equivalents, Sigma, USA) in DMF, was then added and the flask was stirred for 24 hours. To verify if the coupling reaction had reached completion, the TNBS test was performed (negative test outcome). The Fmoc deprotection and coupling steps were repeated until 5 amino acid residues (GRGDS) were coupled to the resin. To conjugate the maleimide linker to the deprotected N-terminus of the peptide, 4 equivalents of maleimidopropionic acid (TCI America, USA) and 12 equivalents of diisopropylcarbodiimide (Sigma, USA) were pre-mixed in dichloromethane (Sigma, USA) for 45 minutes and then added to the SPS flask and stirred for 24 hours. The maleimide-modified GRGDS (mal-GRGDS) sequence was then cleaved from the resin using 95% trifluoroacetic acid (Caledon, Canada) in water. The peptide was allowed to precipitate in cold diethyl ether for 30 minutes. Then, the precipitate was recovered by centrifugation. Finally, the product was purified by HPLC (Shimadzu, Japan) in a C18, 250 x 10 mm, 5 μ m, 100Å column. A mobile phase gradient from 5 % to 20 % (Acetonitrile (w/ 0.1 % TFA):ddH₂O (w/ 0.1 % TFA)) over

30 minutes was performed. A 90% yield was obtained and the product was confirmed by ^1H NMR and mass spectrometry (MS).

3.3 Immobilization of Mal-GRGDS Peptide on Furan-GG Hydrogel

Immobilization of maleimide-containing GRGDS (mal-GRGDS) to furan-modified gellan gum was performed by via Diels-Alder chemistry between the maleimide functional group of the peptide with the furan group of the gellan gum. Furan-GG was first dissolved in MES buffer (100 mM, pH 5.5) at 4 mg/ml and 37 °C and then incubated with mal-GRGDS in a 1:5 furan-maleimide molar ratio for 48h under constant stirring. The solution was then dialyzed (M_w cutoff 12-14kDaltons, Spectrum Labs, USA) alternately against distilled water and PBS (0.1M and pH 7.2) for 5 days. Finally, the water was removed by lyophilization to obtain GRGDS-modified Gellan Gum (GRGDS-GG) as a white powder. The amount of peptide immobilized on the hydrogel was calculated by amino acid analysis.

4. Physicochemical Characterization Techniques

4.1. Scanning Electron Microscopy

Scanning electron microscopy (SEM) provides images of the surface of a given sample by scanning it with a high-energy beam of electrons. SEM was used for analysis of morphology/porosity of the different SPCL tubular scaffolds. For this purpose, all samples were coated with Au/Pd via ion-sputtering prior to observation in a Stereoscan 360 scanning electron microscope (Leica Cambridge Co., Cambridge, United Kingdom).

4.2. Micro-Computed Tomography

Micro-computed tomography (μ -CT) provides valuable information on the 3D morphology of a chosen sample. Some of its advantages includes its non-destructive and the possibility to assess in a quantitative way parameters such as porosity, pore size and interconnectivity [20]. The architecture and porosity of the tubular scaffolds was analyzed by μ -CT using a desktop micro CT scanner (SkyScan 1072, Belgium) at a voltage of 40 kV and a current of 248 μ A. Isotropic slice data were obtained by the system and reconstructed into 2D XY slice images. Around 600 slice images per sample were compiled and subsequently employed in the rendering of 3D XYZ images in order to obtain quantitative architectural parameters. A μ CT analyzer and a μ -CT volume realistic 3D Visualization software, from SkyScan (Belgium), was used as an image processing tool for reconstruction and creation/visualization of 3D scaffold representations.

4.3. Dynamic Mechanical Analysis

Dynamic mechanical analysis (DMA) is a technique used to investigate the viscoelastic properties of polymeric materials. DMA measures the dynamic response of a particular polymeric system, being particularly adequate to evaluate the performance of biomaterials when working under the cyclic solicitations generated by the human body physiological movements [21]. Tubular scaffolds analysis was carried out using a uniaxial compression loading scheme at 37° C, under both dry and wet (Phosphate Buffered Saline - PBS 0.1M and pH 7.2) conditions. DMA was performed in a Triton 2000B equipment (Triton Technology Ltd, Nottinghamshire, United Kingdom).

Scaffolds were subjected to compression cycles of increasing frequencies ranging from 0.1 and 70 Hz with constant amplitude displacements of 0.1 mm.

4.4. Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin. ^1H NMR is the application of nuclear magnetic resonance in NMR spectroscopy with respect to hydrogen nuclei within the molecules of a substance. This is a nondestructive technique that allows determining the structure of organic compounds [22]. Simple NMR spectra are recorded in solution, and solvent protons must not be allowed to interfere. In this thesis, ^1H NMR was employed in chapter VI to analyze the degree of furfurylamine substitution in the gellan gum hydrogel. For this propose, gellan gum was dissolved in deuterated water (D_2O) and the ^1H NMR spectra were obtained with a Varian Mercury-400 MHz NMR spectrometer (Palo Alto, USA). The one-dimensional ^1H NMR spectra were acquired at 25°C, using a 45° pulse, a spectral width of 6.4 kHz and an acquisition time of 2.992 seconds.

4.5. High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) is a highly improved form of column chromatography that is used in biochemistry and analytical chemistry to identify, quantify, and purify the individual components of a solution. Compounds are

separated by injecting a plug of the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their interaction behavior between the mobile liquid phase and the stationary phase. HPLC was used in chapter VI to purify the GRGDS peptide. The product was purified in a Shimadzu equipment (Japan) with a C18, 250 x 10 mm, 5 μ m, 100Å column. A mobile phase gradient from 5 % to 20 % (Acetonitrile (w/ 0.1 % TFA):ddH₂O (w/ 0.1 % TFA)) over 30 minutes was performed, and a 90% yield was obtained.

4.6. Amino Acid Analysis

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. It involves the hydrolyzation of the protein/peptide to its individual amino acid constituents and the samples are typically derivatized for analysis. This technique was employed in chapter VI to calculate the amount of GRGDS immobilized on the gellan gum, using a Waters Pico-Tag System. Samples were dried in pyrolyzed borosilicate tubes in a vacuum centrifugal concentrator and subjected to vapour phase hydrolysis by 6N HCl with 1% phenol at 110°C for 24 hours under pre-purified nitrogen atmosphere. After hydrolysis, excess HCl was removed by vacuum, hydrolyzates washed with redrying solution and derivatized with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acids. The derivatization method is accomplished at room temperature. Derivatized amino acids were redissolved in phosphate buffer and quantified using reverse phase HPLC.

5. Cell Isolation and Expansion

5.1. Rat Lung Fibroblast Cell Line

The rat lung fibroblasts cell line L929, acquired from the European Collection of Cell Cultures (ECACC), was used for the cytotoxicity tests described on chapter III. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% of an antibiotic–antimycotic mixture (Sigma). For the MEM extraction tests, cells were seeded in 12 well plates ($n=3$, 1×10^5 cells/well), while for the MTS test cells were seeded in 96 well plates ($n=3$, 1.8×10^4 /well). In both cases cells were incubated for 24h at 37°C, in a 5% (v/v) CO₂ cell culture incubator.

5.2. Human Oligodendrocyte Cell Line

On chapter III, human oligodendrocyte cell line MO3-13 was encapsulated within the gellan gum hydrogel. For this purpose, cells were first grown as monolayer cultures in DMEM (Sigma) supplemented with 10% FBS (Gibco) and 1% antibiotic–antimycotic mixture (Sigma) until confluence. Trypsin (Sigma) was used to detach the cells from the culture flasks before the experiments were conducted.

5.3. Olfactory Ensheathing Cells

Olfactory ensheathing cells (OECs) were harvested from olfactory bulbs of 4-days-old Wistar rats, according to the protocol described by Ramon-Cuétó *et al.* [23]. Briefly, upon dissection all meninges were removed and the tissue was digested with 0,125%

Collagenase (Sigma) for 20h at 37 °C. The digested tissue was mechanically dissociated with a pipette and then filtrated through a 70 µm cell strainer (BD Falcon). After centrifugation at 1000rpm for 10 min, cells were resuspended and plated in uncoated plates for 18h. A posterior change to uncoated plates for 36h was made, as it is expected that most of the fibroblasts and astrocytes will attach in the first and second period, respectively. Finally, cell suspensions were transferred to poly-D-lysine treaded flasks and cultured in DMEM/F12 (Gibco) with 10% of FBS (Gibco) and 1% of antibiotic-antimycotic solution (Sigma) at 37 °C and 5% CO₂ (v/v). OECs were then enriched by the supplementation with Bovine Pituitary Extract (5µg/ml, Gibco) and Forskolin (2µg/ml, Sigma). These cells were used in the work described on chapters V, VI, and VII.

5.4. Schwann Cells

Schwann cells (SCs) were obtained from sciatic nerves of 4-weeks-old male Wistar rats, according to the protocol described by Mauritz et al. [24] Upon dissection, sciatic nerves were cut into 3 fragments each and incubated in DMEM-F-12 (Gibco), with 10% Fetal Bovine Serum (FBS, Gibco), 1% Penicillin-Streptomycin antibiotic (Pen-Strep, Gibco), Forskolin (2µg/ml, Sigma) and Bovine Pituitary Extract (5µg/ml, Gibco). Following 7 days of incubation at 37°C and 5% CO₂, the nerve fragments were enzymatically dissociated with Collagenase solution (320U/ml) (Sigma) containing 5U/ml of DNase (Worthington) for 20 hours. After mechanical dissociation, the collagenase was removed by repeated centrifugations. Cells were then allowed to grow in Poly-D-Lysine coated flasks. These cells were used in the work described on chapter V.

5.5. Bone Marrow Stromal Cells

Bone Marrow Stromal Cells (BMSCs) acquired from Lonza (Switzerland), were cultured in α -MEM (Invitrogen/Gibco) supplemented with 10% FBS and 1% antibiotic-antimycotic mixture. The culture medium was changed every 2/3 days. Upon confluence, cells were trypsinized and passaged to new T75 flasks. These cells were used in the work described on chapter VII.

5.6. Human Umbilical Cord Perivascular Cells

Human umbilical cord perivascular cells (HUCPVCs) were isolated according to the procedure originally described by Sarugaser *et al.* [25]. Pieces of cord were dissected by first removing the epithelium of the umbilical cord section along its length to expose the underlying Wharton's jelly. Each vessel, with its surrounding Wharton's Jelly matrix, was then pulled away, after which the ends of each dissected vessel were tied together with a suture creating "loops" that were placed into a solution of 0.5–0.75 mg/mL collagenase (Sigma) with phosphate-buffered saline (PBS, Gibco). After 18 h, the loops were removed from the suspension, which was then diluted with PBS to reduce the viscosity of the suspension and centrifuged. Following the removal of the supernatant, cells were resuspended in culture media, α -MEM (Gibco) supplemented with 10% FBS (Gibco) and 1% antibiotic/antimycotic (Sigma), counted using a hemocytometer and plated out in T75 flasks at a density of 4,000 cells/cm². The culture medium was changed every 2/3 days. Upon confluence, cells were trypsinized and passaged to new T75 flasks. These cells were used in the work described on chapter VII.

5.7. Human Adipose Stem Cells

Human adipose stem cells (ASCs) were a kind gift from Prof. Jeff Gimble from the Pennington Biomedical Research Center on Baton Rouge, USA. The cells were isolated as previously described [26]. These cells were used in the work described on chapter VII.

5.8. Neural Stem Cells

Neural stem cells (NSCs) were isolated from the subependymal region of the lateral ventricles in the forebrain of adult male Wistar rats as previously described [27]. Cells were grown in neurobasal medium (Invitrogen), with 2% of B27 neural supplement (Invitrogen), 1% of L-glutamine (Sigma), 1% of penicillin–streptomycin (Sigma), 20 ng/ml EGF (recombinant human EGF; Invitrogen), 20 ng/ml bFGF (recombinant human bFGF; Invitrogen) and 2 ng/ml heparin (Sigma). Cell number and viability were determined with a hemocytometer and the trypan blue exclusion test. Dissociated cells were plated in complete media and incubated in a humidified atmosphere at 37 °C with 5% of CO₂. Neurospheres were observed within 2–3 weeks, after which cells were passaged weekly. These cells were used in the work described on chapter VI.

6. Cell/Materials Experiments

6.1. Seeding of OECs or SCs on SPCL Scaffolds

To ensure adherence of cells, SPCL scaffolds were coated with poly-D-lysine before cell seeding. Cells were centrifuged at 1200 rpm for 5 min and then resuspended in a

small volume of medium to yield a solution with a density of 5×10^4 cell/10 μ l. Subsequently, scaffolds were transferred to empty wells and 10 μ l of cell suspension were dropped on the top of the inner lumen of the semi tubular scaffolds. After an incubation of 2 h in a humidified atmosphere at 37 °C and 5% CO₂, SPCL scaffolds were transferred to new wells containing complete medium (for composition details refer to OECs or SCs cell culture section) and incubated for 2, 7, 14 and 21 days for the *in vitro* assays. This proceeding was used in the work described in chapter V.

6.2. Encapsulation of Oligodendrocytes in Gellan Gum Hydrogel

In the work described in chapter III, the oligodendrocyte cell line M03 was encapsulated in 1% (w/v) of gellan gum hydrogel. Cells and hydrogel were prepared as described above (section 5.2 and 3.2, respectively). Then, cells were resuspended (20×10^4 cells/ml) in the gellan gum solution and injected into central canal of SPCL tubular scaffolds. The Cell/Scaffolds constructs were then allowed to rest at room temperature for 3 minutes to form a solid gel. Then, constructs were incubated in standard culture medium DMEM (Gibco) with 10% of FBS (Gibco) and 1% of antibiotic/antimycotic (Sigma) for 7 days at 37° C in a 5% (v/v) CO₂ cell culture incubator. For this period, the distribution of viable cells was evaluated by confocal microscopy (Fluoview FV 1000, Olympus) in combination with calcein-AM staining (Sigma).

6.3. Neural Stem Cells Culture in GRGDS-Gellan Gum Hydrogel

In the work described in chapter VI, bioactivity of GRGDS was assessed by the analysis of NSCs growth, morphology and differentiation when cultured on the modified gellan

gum hydrogel for 2 and 7 days. NSCs were mechanically dissociated into a single cell suspension and either seeded on the surface or encapsulated into GRGDS-GG hydrogel in complete medium (as described in Section 5.8). The cell density was 2×10^4 cells/cm² and hydrogels were incubated in a humidified atmosphere at 37 °C and 5% of CO₂. Furan-modified GG was used as a control. Cell growth and morphology was evaluated following phalloidin/DAPI staining and differentiation following immunocytochemistry (ICC). Analysis was carried out using a Zeiss Observer Z1 microscope with a Yokogawa confocal scan unit; images were captured and processed using Volocity 4.3.2 software.

6.4. OECs and NSCs Co-Culture in GRGDS-Gellan Hydrogel

In the work described in chapter VI, it was evaluate the potential synergistic or antagonistic effects between OECs and NPCs within the GRGDS-GG hydrogel. OECs (1×10^5 cells/cm²) and NPCs (4×10^4 cells/cm²), obtained as described in section 5, were encapsulated together in the GRGDS-GG hydrogel. Both cells were pre-labeled before encapsulation. A green tracer was used for OECs labeling (C34554, Invitrogen) and NPCs were labeled with a far red tracer (C34553, Invitrogen). Both labeling protocols were performed according to the manufacturer instructions. Cell interactions and growth were then analyzed by confocal analyses after 7 days of culture.

7. Co-Cultures Studies

7.1. OECs and NSCs Co-Culture

To evaluate the potential beneficial or detrimental effects between OECs and NPCs, direct and transwell co-culture experiments were performed. OECs (1×10^5 cells/cm²) and NSCs (4×10^4 cells/cm²), obtained as described in section 5, were seeded either simultaneously on the same fibronectin coated cover glass or in indirect contact with OECs cultured on the fibronectin coated cover glass and NSCs on the transwell. Cells were allowed to grow in complete NSCs culture medium (see section 5.8) for 24h and then cultured in the absence of the growth factors FGF and EGF. In the direct co-culture experiment, in order to clearly identify one cell population from another, OECs were labeled (according to manufacturer instructions) with a cell tracing reagent (C34554, Invitrogen) before seeding. After 7 days of incubation, cell growth and NSCs differentiation was assessed by ICC. Analysis was performed using an Olympus BX61 fluorescence microscope. OECs and NSCs cultured alone were used as controls. This work is described in chapter VI.

7.2. OECs and Mesenchymal Stromal Cells Co-Culture

In the work described in chapter VII, potential positive or negative effects of the cell secretome of OECs and bmMSCs, HUCPVCs or ASCs was evaluated using a transwell system. OECs were seeded (1×10^5 cells/cm²) on a poly-D-lysine coated well and MSCs were seeded (4×10^4 cells/cm²) on the transwell. Cell proliferation, metabolic activity and differentiation were assessed after 2 and 7 days in culture. ICC analysis was performed using an Olympus BX61 fluorescence microscope. OECs and Mesenchymal

stromal cells cultured alone were used as controls. Cells were obtained and maintained as described in section 5 of this chapter.

8. *In Vitro* Biological Testing

8.1. MTS Test

MTS test is based on the bioreduction of the substrate, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (Promega), into a brown formazan product by dehydrogenase enzymes in metabolically active cells, and is commonly used for cell viability evaluation. This test was used in the work described on chapter III to assess the possible cytotoxicity of SCPL/Gellan Gum constructs leachables. Moreover, MTS test was also used in the work described on chapter V and VI to assess the metabolic activity of cells after seeding in SPCL scaffolds or after co-cultures studies. After each specific time point, complete culture medium was replaced by serum-free medium containing MTS in a 5:1 ratio and incubated in a humidified atmosphere at 37 °C and 5% CO₂. After 3h of incubation, the optic density for triplicates of each sample (n=3) was measured at 490 nm in a microplate reader.

8.2. Minimum Essential Medium Extraction Test

In the work described in chapter III, the cytotoxicity of the developed scaffolds was assessed using MTS and minimum essential medium (MEM) extraction tests. The objectives of the MEM extraction tests are to evaluate changes in cell morphology and/or cell growth inhibition derived from possible toxic leachables released from the scaffolds. The test was performed according to ISO/EN 10993 part 5 guidelines [28].

Briefly, leachable-conditioned medium was collected after 7, 14, 21, and 28 days of incubation at 37°C. The ratio material weight to extract fluid was constant and equal to 0.2g/ml for porous samples, while for latex the ratio of material outer surface to extraction fluid was 2.5 cm²/ml. After each period the extracts were filtered through a 0.45 µm pore size filter. Latex extracts were used as negative controls for cell viability, while standard culture medium was used as positive control for cell viability. The cell line L929, expanded and cultured was described in section 5.1, was used in this test. After 24h of culture, the standard culture medium was removed from the wells and an identical volume, 1 ml, of extraction fluid was added. Cultures were then incubated with the extracts for 72h, after which it was conducted a live/death assay by staining live cells with calcein-AM (1mg/ml; Molecular Probes) and non-viable cells with propidium iodide (PI, 0.1 mg/ml, Molecular Probes). Upon staining, cultures were observed under a fluorescence microscopy (Olympus BX-61).

8.3 DNA Quantification

The accurate determination of DNA concentration is essential for many processes in molecular biology and physiology and includes both gel- and cuvette-based methods. The recently introduced fluorescent dye, PicoGreen, has several advantages over other methods due to its sensitivity and specificity for double-stranded DNA (dsDNA) [29]. In the work described in chapter V, PicoGreen fluorescent assay was used to calculate cell proliferation. The samples were collected on each specific time point, washed twice with a sterile PBS solution and transferred into microtubes containing 1 mL of ultra-pure water. Samples were incubated for 1 hour at 37°C in a water-bath and then stored in a -80°C freezer until testing. Prior to DNA quantification constructs were thawed and

sonicated for 15 minutes. Samples and standards (ranging between 0 and $2\mu\text{g.mL}^{-1}$) were prepared per each well of an opaque 96-well plate were added $28.7\mu\text{L}$ of sample or standard plus $71.3\mu\text{L}$ of PicoGreen solution and $100\mu\text{L}$ of Tris-EDTA buffer. Triplicates were made for each sample or standard. The plate was incubated for 10 minutes in the dark and fluorescence was measured on a microplate reader (BioTek) using an emission of 490 nm and an absorbance wavelength of 520 nm. A standard curve was created and sample DNA values were read off from the standard graph.

8.4. Cell Proliferation ELISA, BrdU

The colorimetric 5-bromo-2'-deoxyuridine (BrdU) assay (Roche) was employed in the work described in chapter VII, with the aim of quantify the DNA synthesis during cell activation and proliferation. This ELISA assay measures proliferative cells by quantitating BrdU incorporated into newly synthesized DNA of replicating cells. It is a nonradioactive alternative to the $[^3\text{H}]$ -thymidine-based cell proliferation assay with comparable sensitivity. To perform the assay, BrdU was added to the cell cultures for 24 hours and after that the medium was removed. The cells were then fixed and its DNA denatured with FixDenat (Roche) in a single step, after which the anti-BrdU peroxidase antibody (Roche) was added. The immune complexes were detected by the quantification of the substrate reaction product, measuring the optical density at 370 nm (reference filter set at 492 nm) in a multiplate reader (BioRad).

8.5. Phalloidin/DAPI Staining

In the work described in chapter V, VI and VII, cell adhesion, morphology and distribution were assessed using a phalloidin/DAPI staining protocol. Phalloidin is a heptapeptide toxin, produced by poisonous mushroom *Amanita phalloides*, that binds tightly and specifically to polymerized actin filaments. For this reason, biotinylated and fluorescent phalloidins are used in various analysis techniques to label, identify, quantify and stabilize F-actin in fixed and permeabilized tissue sections, cell cultures or cell free experiments [30]. In contrast, DAPI (diamidino-phenylindole, dihydrochloride) is a popular nuclear counterstain for the use in multicolor fluorescent techniques. Its blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes of others structures. To perform the phalloidin/DAPI staining protocol, cells were fixed with 4% of PFA for 30 min at room temperature and then treated with 0.3% TritonX-100. After washing several times with PBS, 0.1 µg/ml of phalloidin (Sigma) was added to the cells for 30 min. Finally, cell nuclei were counterstained with DAPI (1 µg/ml, Invitrogen) for 10 min.

8.6. Immunocytochemistry

Immunocytochemistry (ICC) is a microscopic method for determining the presence, subcellular localization, and relative abundance of an antigen of interest, most commonly a protein, in cultured cells [31]. This technique was used in the work described in chapter V, VI and VII, being employed the following antibodies: monoclonal rabbit anti- β -III tubulin (Chemicon) for neurons identification; monoclonal mouse anti-GFAP (Chemicon) for astrocytes; monoclonal mouse anti-O4 (R&D Systems) for oligodendrocytes; monoclonal mouse anti-Nestin (Millipore) for

progenitor cells; polyclonal rabbit anti-p75 (Millipore) for OECs; and monoclonal rabbit anti-S100 (Invitrogen) for OECs and SCs. For all immunohistochemical procedures, the appropriate controls were obtained by omission of the relevant primary antibody. Cells on the substrates were fixed with PBS solution containing 4% PFA for 20 min (on glass) or 1 h (on the hydrogel) at room temperature and then washed with PBS. After cell membrane permeation (except for p75, O4 and S100 antibodies) and blocking by treating with 0.3% Triton X-100 (Sigma) and 10% of FBS solution at room temperature for 1 h, each specific primary antibody solution was added for 1 h. After washing with 0.5% of FBS in PBS, the samples were exposed to the specific secondary antibody (Invitrogen) for 1h and then washed with 0.5% FBS. Finally, cell nuclei were counterstained with 1 µg/ml DAPI (Invitrogen) for 1h.

9. *In Vivo* Testing

9.1. Animals

Eight weeks old male Wistar rats (Charles River), housed in light and temperature controlled rooms and fed with standard diet, were used in the studies described in chapter III and V. The Animal Care Committee of the Research Institute approved the animal protocols in accordance with standardized Animal Care Guidelines [32]. Handling was performed for 3 days before the surgery. Following SCI surgery rats were kept under heat lamps and received vitamins and antibiotics. Bladder evacuation was done manually. Throughout the treatment and recovery period, animals were examined for symptoms of illness or potential reaction to the treatment.

9.2. Spinal Cord Injury Surgery

All animals were anesthetized by intraperitoneal injection of a mixture (1,5:1) of ketamine (60 mg/ml, Imalgen, Merial) and medetomidine hydrochloride (0.4 mg/ml, Dorbene Vet, Laboratorios SYVA S.A.). Once anaesthetized, fur was shaved from the surgical site and the skin disinfected with chlorohexidine (AGB). Then a dorsal midline incision was made from T6–T11 and the paravertebral muscles retracted. A laminectomy was performed at the junction T8–T9 in which the spinous processes were removed and the spinal cord exposed. Two hemisections were performed, 3 mm apart on the left side, and the tissue in between removed. After scaffolds implantation, paravertebral muscles and skin were separately closed with Vicryl sutures (Johnson and Johnson). The incision of control animals was closed after SCI, without scaffold implantation.

In the work described in chapter III this surgery was used in order to study the histocompatibility of scaffolds developed. SPCL/gellan gum constructs were implanted in the defect site, with the SPCL phase aligned with the vertebral bone while the gellan gum was facing, and filling, the injured spinal cord.

In the work described in chapter V, this surgery was employed in order to study the effects of spine stabilization on motor behavior. After SCI, SPCL scaffolds were implanted at the vertebral bone level, juxtaposed to the spinal cord. Bone cement (Biomet, USA) was used to fix the scaffolds margins to bone. Animals were divided into 2 experimental groups: animals with spine stabilization through SPCL scaffolds implantation (SPCL, n=9) and animals without spine stabilization (SCI, n=5). A third group of animals, subjected to laminectomy only, were used as controls (Sham, n=5).

9.3. Subcutaneous Implantation of SPCL/Gellan Gum Constructs – Biocompatibility Assays

All animals were anesthetized by intraperitoneal injection of a mixture (1,5:1) of ketamine (60 mg/ml, Imalgen, Merial) and medetomidine hydrochloride (0.4 mg/ml, Dorbene Vet, Laboratorios SYVA S.A.). Once anaesthetized, four incisions were performed (reaching a maximum of 1.5 cm each) being two in the interscapular region and another two in the lumbar region. With the help of a forceps two side pockets were created through each of the incisions and SPCL/gellan gum scaffolds were subcutaneously implanted. Four scaffolds were implanted per animal, being two on the anterior region and other two on the posterior region. The incision sites were sutured and the rats transferred to heating recovery compartments and when the recovery from anesthesia was confirmed they were returned to their respective compartments and kept under food and drink *ad libitum*. The hybrid structures stayed implanted subcutaneously in the back of the animals for 7, 14, 21 and 28 days.

9.4. Motor Behavior Analysis by BBB Score

In the work described in chapter V, the motor behavior of all rats were assessed with the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) [33] on day 3 and 2, 5, 7, 9, 12 weeks after injury. The BBB is a 21-point scale designed to assess hindlimb locomotor recovery following thoracic spinal cord injury. A BBB score of 0 indicates no hindlimb movement. A BBB score of 1 through 8 indicates joint movement, but no weight support. A BBB score of 9 through 20 indicates an ability to support weight and use the limb for locomotion but with some degree of abnormality. A BBB score of 21

corresponds to the locomotion of a normal rat. Detailed information can be found in Table 1.

Table II.1. Basso, Beattie, Bresnahan Locomotor Rating Scale [33]

0	No observable hindlimb movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e. when stationary) or occasional, frequent or consistent weight-supported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps; no FL/HL coordination
11	Frequent to consistent weight-supported plantar steps and no FL/HL coordination
12	Frequent to consistent weight-supported plantar steps and occasional FL/HL coordination
13	Frequent to consistent weight-supported plantar steps and frequent FL/HL coordination
14	Consistent weight-supported plantar steps; consistent FL/HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL/HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL/HL coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
17	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift-off
18	Consistent plantar stepping and consistent FL/HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
19	Consistent plantar stepping and consistent FL/HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact and lift-off, and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift-off, and trunk instability; tail consistently up
21	Consistent plantar stepping and consistent gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

9.5. Open Field Test

The open field test is commonly used to assess exploratory and anxiety-like behavior in laboratory animals. Moreover, the open field is also a versatile test that allows the assessment of locomotor behavior by measuring the amount of rearing activity and the total distance travelled by the animals [34]. The test was performed in an arena (43.2 cm × 43.2 cm) with transparent acrylic walls (Med Associates Inc.) placed in a brightly illuminated room. Animals started the test at the arena's centre and were given 5 min to explore it. Total distance travelled in the arena and number of rearings was automatically registered by equipment sensors. This test was used in the work described in chapter V.

9.6. RotaRod Test

The rotarod test is used to assess motor coordination and balance in rodents. Animals have to keep their balance on a rotating rod. It is measured the time (latency) it takes the rat to fall off the rod, rotating at different speeds or under continuous acceleration. The work described in chapter V was performed in rotarod equipment from TSE systems. Each animal was placed on a 10-cm diameter, 15-cm long rod, rotating at constant speed. Impairment of motor coordination was defined as the inability of rats to remain on the rotating rod for a 60s test period. Experimentally animals were pre-trained on the rotating track 24 h before the proper test. The protocol consisted of 3 days of testing at 4, 8 and 12 rpm in, respectively, 1st, 2nd and 3rd day for a maximum of 60s in four trials, with a 10 min interval between each trial. The latency to fall (in seconds) was recorded by equipment sensors.

9.7. Tissue Preparation

In the work described in chapter III and V, and after each defined time point, rats were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (Ceva Saude Animal). Then, animals were perfused through the ascending aorta with 4% paraformaldehyde in PBS. A 2.5-3 cm length of spine and spinal cord, centered on the site of hemisection and scaffolds placement, was carefully removed and fixed in neutral buffered formalin. After decalcification, spinal cord and spine were carefully spitted being then embedded in paraffin and processed for hematoxylin–eosin staining.

9.8. Hematoxylin-Eosin Staining

Hematoxylin-Eosin (HE) stain is a popular staining method in histology. It is the most widely used stain in medical diagnosis. This protocol can be employed in a variety of fixed tissues and displays a broad range of cytoplasmatic, nuclear, and extracellular matrix (ECM) features. In a typical tissue, nuclei are stained bleu by hematoxylin, whereas cytoplasm and ECM are colored in various shades of red, pink and orange by eosin [35]. In the work described in chapter III and V, histological processing was conducted by dehydrating the samples in increasing ethanol concentrations, embedding them in paraffin and cutting for posterior analysis using a microtome (Leica RM2155). HE staining was performed immersing tissue sections in hematoxylin and eosin solutions using an automatic processor (Leica TP1020-1). The slides were then washed in distilled water, dehydrated and finally cleared in xylene substitute and mounted using Microscopy Entellan® (Merk & Co., Inc.).

10. Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences among groups were assessed by one way ANOVA test by the two-way ANOVA test followed by the Tukey post-hoc test or by the Bonferroni post-hoc test. A p-value of ≤ 0.05 (95% confidence level) was set as the criteria for statistical significance.

References

- [1] Han J-A, Lim S-T. Structural changes of corn starches by heating and stirring in DMSO measured by SEC-MALLS-RI system. *Carbohydrate Polymers*. 2004;55:265-72.
- [2] Funami T, Kataoka Y, Omoto T, Goto Y, Asai I, Nishinari K. Food hydrocolloids control the gelatinization and retrogradation behavior of starch. 2b. Functions of guar gums with different molecular weights on the retrogradation behavior of corn starch. *Food Hydrocolloids*. 2005;19:25-36.
- [3] Gomes ME, Godinho JS, Tchalamov D, Cunha AM, Reis RL. Alternative tissue engineering scaffolds based on starch: processing methodologies, morphology, degradation and mechanical properties. *Materials Science and Engineering: C*. 2002;20:19-26.
- [4] Marques AP, Reis RL, Hunt JA. An In Vivo Study of the Host Response to Starch-Based Polymers and Composites Subcutaneously Implanted in Rats. *Macromolecular Bioscience*. 2005;5:775-85.
- [5] Salgado AJ, Coutinho OP, Reis RL. Novel Starch-Based Scaffolds for Bone Tissue Engineering: Cytotoxicity, Cell Culture, and Protein Expression. *Tissue Engineering*. 2004;10:465-74.
- [6] Marques AP, Reis RL. Hydroxyapatite reinforcement of different starch-based polymers affects osteoblast-like cells adhesion/spreading and proliferation. *Materials Science and Engineering: C*. 2005;25:215-29.

- [7] Ciardelli G, Chiono V, Vozzi G, Pracella M, Ahluwalia A, Barbani N, et al. Blends of Poly-(ϵ -caprolactone) and Polysaccharides in Tissue Engineering Applications. *Biomacromolecules*. 2005;6:1961-76.
- [8] Li W-J, Danielson KG, Alexander PG, Tuan RS. Biological response of chondrocytes cultured in three-dimensional nanofibrous poly(ϵ -caprolactone) scaffolds. *Journal of Biomedical Materials Research Part A*. 2003;67A:1105-14.
- [9] Li W-J, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, et al. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. *Biomaterials*. 2005;26:599-609.
- [10] Moorhouse R, Colegrove G T, Sandford P A, Baird J K, Kang K S. PS-60: A New Gel-Forming Polysaccharide. *Solution Properties of Polysaccharides: American Chemical Society*; 1981. p. 111-24.
- [11] Oliveira JT, Reis RL. Polysaccharide-based materials for cartilage tissue engineering applications. *Journal of Tissue Engineering and Regenerative Medicine*. 2011;5:421-36.
- [12] Pszczola DE. Gellan gum wins IFT's Food Technology Industrial Achievement Award. *Food Technol*. 1993;47 94-6.
- [13] Oliveira JT, Gardel LS, Rada T, Martins L, Gomes ME, Reis RL. Injectable gellan gum hydrogels with autologous cells for the treatment of rabbit articular cartilage defects. *Journal of Orthopaedic Research*. 2010;28:1193-9.
- [14] Oliveira JT, Martins L, Picciochi R, Malafaya PB, Sousa RA, Neves NM, et al. Gellan gum: A new biomaterial for cartilage tissue engineering applications. *Journal of Biomedical Materials Research Part A*. 2010;93A:852-63.
- [15] Yeong W-Y, Chua C-K, Leong K-F, Chandrasekaran M. Rapid prototyping in tissue engineering: challenges and potential. *Trends in Biotechnology*. 2004;22:643-52.
- [16] Leong KF, Cheah CM, Chua CK. Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs. *Biomaterials*. 2003;24:2363-78.
- [17] Kolb HC, Finn MG, Sharpless KB. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angewandte Chemie International Edition*. 2001;40:2004-21.
- [18] Diels O, Alder K. Synthesen in der hydroaromatischen Reihe. *Justus Liebigs Annalen der Chemie*. 1928;460:98-122.

- [19] Wellings DA, Atherton E. Standard Fmoc protocols. In: Gregg BF, editor. *Methods in Enzymology*: Academic Press; 1997. p. 44-67.
- [20] Ho ST, Hutmacher DW. A comparison of micro CT with other techniques used in the characterization of scaffolds. *Biomaterials*. 2006;27:1362-76.
- [21] Mano JF, Vaz CM, Mendes SC, Reis RL, Cunha AM. Dynamic mechanical properties of hydroxyapatite-reinforced and porous starch-based degradable biomaterials. *Journal of Materials Science: Materials in Medicine*. 1999;10:857-62.
- [22] Hornak JP. e-book: *The Basics of NMR* In: Hornak JP, editor.: Rochester Institute of Technology; 1999.
- [23] Ramón-Cueto A, Cordero MI, Santos-Benito FF, Avila J. Functional Recovery of Paraplegic Rats and Motor Axon Regeneration in Their Spinal Cords by Olfactory Ensheathing Glia. *Neuron*. 2000;25:425-35.
- [24] Mauritz C, Grothe C, Haastert K. Comparative study of cell culture and purification methods to obtain highly enriched cultures of proliferating adult rat Schwann cells. *Journal of Neuroscience Research*. 2004;77:453-61.
- [25] Sarugaser R, Ennis J, Stanford WL, Davies JE. Isolation, Propagation, and Characterization of Human Umbilical Cord Perivascular Cells (HUCPVCs). In: Audet J, Stanford WL, editors. *Methods in Molecular Biology*: Humana Press Inc; 2009. p. 269-79.
- [26] Dubois SG, Floyd EZ, Zvonic S, Kilroy G, Wu X, Carling S, et al. Isolation of human adipose-derived stem cells from biopsies and liposuction specimens. In: Prockop DJ, Phinney DG, Bunnell BA, editors. *Methods in Molecular Biology*: Springer; 2008. p. 69-79.
- [27] Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, et al. Neural stem cells in the adult mammalian forebrain: A relatively quiescent subpopulation of subependymal cells. *Neuron*. 1994;13:1071-82.
- [28] ISO10993. Test for cytotoxicity: In vitro methods. *Biological compatibility of medical devices*, 1992.
- [29] Schofield GG. PicoGmeter, a custom-made fluorometer for the quantification of dsDNA by PicoGreen (R) fluorescence. *Biotechniques*. 2004;37:778-82.
- [30] De La Cruz EM, Pollard TD. Kinetics and thermodynamics of phalloidin binding to actin filaments from three divergent species. *Biochemistry*. 1996;35:14054-61.
- [31] Burry RW. Immunocytochemistry. In: Burry RW, editor. *A Practical Guide for Biomedical Research*. New York: Springer; 2010.

- [32] Van Zutphen LFM, Baumans V, Beynen, AC. Principles of Laboratory Animal Science: Elsevier; 2001.
- [33] Basso DM, Beattie MS, Bresnahan JC. A Sensitive and Reliable Locomotor Rating-Scale for Open-Field Testing in Rats. *Journal of Neurotrauma*. 1995;12:1-21.
- [34] Sousa N, Almeida OFX, Wotjak CT. A hitchhiker's guide to behavioral analysis in laboratory rodents. *Genes, Brain and Behavior*. 2006;5:5-24.
- [35] Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and Eosin Staining of Tissue and Cell Sections. *Cold Spring Harbor Protocols*. 2008;2008:pdb.prot4986.

SECTION III

CHAPTER III

Development and Characterization of a Novel Hybrid Tissue Engineering Based Scaffold for Spinal Cord Injury Repair

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Development and Characterization of a Novel Hybrid Tissue Engineering Based Scaffold for Spinal Cord Injury Repair.*

Abstract

Spinal cord injury (SCI) represents a significant health and social problem and therefore it is urgent to develop novel strategies that can specifically target it. In this context, the objective of the present work was to develop a new range of 3D tubular structures aimed at inducing the regeneration within SCI sites. Up to six different 3D tubular structures were initially developed by rapid prototyping - 3D bioplotting – based on a biodegradable blend of starch. These structures were then further complemented by injecting Gellan Gum, a polysaccharide based hydrogel, in the central area of structures. The mechanical properties of these structures were assessed by DMA, under both dry and wet conditions, and their morphologies/porosities analysed by micro-CT and SEM. Biological evaluation was carried out for determining their cytotoxicity, using both MEM extraction and MTS tests, as well as by encapsulation of an oligodendrocyte like cell (M03-13 cell line) within the hydrogel phase. The histomorphometric analysis showed a fully interconnected network of pores with porosity ranging from 70% -85%. Scaffolds presented compressive modulus ranging from 17.4 to 62.0 MPa and 4.42 to 27.4 MPa under dry and wet conditions respectively. Cytotoxicity assays revealed that the hybrid SPCL/Gellan Gum scaffolds were non cytotoxic as they did not cause major alterations on cell morphology, proliferation and metabolic viability. Finally, preliminary direct contact assays showed that the hybrid scaffolds could support the *in vitro* culture of oligodendrocyte like cells. Further work will focus on the behaviour of these scaffolds when implanted in SCI animal models.

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1. Introduction

Spinal Cord Injury (SCI) results in a devastating condition with enormous financial [1-4], social and personal costs [5]. It is estimated that the annual incidence of SCI in the United States alone is approximately 40 cases per 1 million. It is commonly characterized by a primary injury that leads to a cascade of cellular and biochemical reactions which cause further damage. The latter is known as "secondary injury" being characterized by microvascular alterations, edema, ischemia, necrosis, free radicals formation, lipid peroxidation, excitatory neurotransmitter accumulation, inflammatory response and other molecular changes contributing to further neural damage [2,6-9].

Current approaches used in clinical practice are mainly based on the use of pharmacological agents, like methylprednisolone [10,11]. However, its use is quite controversial as recent studies failed to reveal conclusive beneficial outcomes [12, 13]. Therefore, it is imperative to find novel therapeutic strategies that can specifically target SCI regeneration.

In recent years, different approaches have been proposed in order to develop valid strategies for SCI repair such as biomolecular [14-17], cellular [18-23] and biomaterial based therapies [24-28]. However, due to the complexity of SCI repair it is unlikely that a single strategy will be adequate to tackle the problem. It is probable that only by following an integrated strategy, such as that presented by Tissue Engineering (TE) [29], it will be possible to develop a successful approach. In this field, it is of general agreement that a scaffold, a 3D biodegradable structure, plays a central role in the aid of the regenerative process. In recent years, natural based biodegradable polymers have

emerged as a possible biomaterial source for scaffold development within the SCI field [28,30-34]. Among these, polysaccharides are particularly appealing due to their structural similarities to components of host tissues. Moreover, they can also be enzymatically degraded in biological systems, allowing for better control of endogenous degradation rate along with other properties [35,36].

Within the present study we aimed at developing a new biphasic tubular scaffold for SCI regeneration. It is comprised of two natural polysaccharide based biomaterials: starch/poly- ϵ -caprolactone blend (SPCL) and gellan gum. SPCL has been originally proposed by Reis and colleagues for a wide range of applications including the culture of neurons, astrocytes and oligodendrocytes [37-43]. Gellan gum is an anionic exocellular polysaccharide secreted from the *Sphingomonas paucimobilis* bacterium. It is composed of repeated tetrasaccharide units of glucose, glucuronic acid, and rhamnose residues in a 2:1:1 ratio [44]. It was originally used as a thickener/gelling agent in food applications, and was recently proposed as a hydrogel for regenerative medicine purposes [44]. The hybrid structures proposed in this report consist of a SPCL semi-rigid tubular porous structure filled by a gellan gum hydrogel concentric core. In this concept the SPCL tubular structure assures mechanical stability to the entire construct, namely by establishing a connection to the adjacent vertebral bone. In this sense the SPCL phase is aimed at mimicking the bone functions while the gellan gum hydrogel is aimed as a cell encapsulation system to support axonal regeneration in the injured spinal cord.

In this report we address the mechanical and preliminary biological characterization of SPCL/gellan gum hybrid tubular structures. Our results reveal that these novel

structures exhibit adjustable mechanical performance and porosity, minimal cytotoxicity and support the encapsulation of oligodendrocyte like cells. Moreover it was also observed that these 3D hybrid structures caused a minimal inflammatory reaction when implanted subcutaneously and in a hemisection spinal cord injury in Wistar rats.

2. Materials and Methods

2.1. Materials

The materials used in this work were a 30/70 (wt%) biodegradable blend of corn-starch with poly- ϵ -Caprolactone (SPCL) and a polysaccharide gellan gum hydrogel 1% (w/v) (Sigma, Saint Louis, USA).

2.2. Development of SPCL tubular scaffolds

The SPCL tubular scaffolds were processed in a two step methodological approach:

- 1) Porous sheets featuring different pore geometries were produced by 3D plotting rapid prototyping (Bioplotter®; Envisiontec GmbH, Marl, Germany) [45,46] using two different inter filament orientations (90° and 45°);
- 2) Tubular scaffolds were obtained by rolling up porous sheets around a cylinder and subsequent heat treatment at 65° C during 30 min for inducing the adhesion between filaments.

Up to six different porous tubular scaffold designs were obtained featuring a single or double layer combined with three different filaments' oriented relative to the tube main axis: filaments oriented at 0° and 90° relative to the tube main axis (90/1-2, further referred as 90/1 and 90/2 for structures with one or two layers), filaments oriented at 0° and 45° relative to the tube main axis (45/1-2, further referred as 45/1 and 45/2), and

filaments oriented at 45° relative to the tube main axis (X/1-2, further referred as X/1 and X/2). As example of the adopted scaffold references, processing combination 90/2 refers to a double layer scaffold featuring filaments' orientated at 0° and 90° relative to the tube main axis, while 45/1 refers to a single layer scaffold featuring filaments' orientated at 45° relative to the tube main axis.

2.3. *Scanning Electron Microscopy*

Scanning electron microscopy (SEM) was used for analysis of morphology/porosity of the different SPCL tubular scaffolds. For this purpose, all samples were coated with Au/Pd via ion-sputtering prior to observation in a Stereoscan 360 scanning electron microscope (Leica Cambridge Co., Cambridge, United Kingdom).

2.4. *Micro-computed tomography*

The architecture and porosity of the tubular scaffolds was analyzed by micro-computed tomography (μ -CT) using a desktop micro CT scanner (SkyScan 1072, Belgium) at a voltage of 40 kV and a current of 248 μ A. Isotropic slice data were obtained by the system and reconstructed into 2D XY slice images. Around 600 slice images per sample were compiled and subsequently employed in the rendering of 3D XYZ images in order to obtain quantitative architectural parameters. A μ CT analyzer and a μ -CT volume realistic 3D Visualization software, from SkyScan (Belgium), was used as an image processing tool for reconstruction and creation/visualization of 3D scaffold representations.

2.5. *Dynamic Mechanical Analysis*

Dynamic mechanical analysis (DMA) was carried out using a uniaxial compression loading scheme at 37° C, under both dry and wet (Phosphate Buffered Saline - PBS 0.1M and pH 7.2) conditions. DMA was performed in a Tritec 2000B equipment (Triton Technology Ltd, Nottinghamshire, United Kingdom). The frequency range used between 1 and 70 Hz. 3 replicates were conducted for each type of scaffold morphology.

2.6. *Development of the 3D Hybrid Systems*

The integration of gellan gum hydrogel into the central canal of the SPCL tubular scaffolds was performed by placing the tubular scaffolds into a specially designed mould, and by filling the empty volume with a gellan gum hydrogel as previously described by Oliveira *et al.* [44].

2.7. *In vitro cytotoxicity assessment*

In order to assess the short-term cytotoxicity of the developed scaffolds, the following guidelines described by Salgado and colleagues were used [47]: MEM extraction and MTS tests, both with 7, 14, 21, and 28 days of extraction period. In all tests the ratio material weight to extract fluid was constant and equal to 0.2g/ml for porous samples, while for latex (negative control for cell viability) the ratio of material outer surface to extraction fluid was 2.5 cm²/ml. After each period the extracts were filtered through a 0.45 µm pore size filter. These assays are particularly suitable for assessing the possible toxic effect of leachables extracted from biomedical polymers. The objectives of the MEM extraction test are to evaluate changes in cell morphology and growth inhibition, whereas the MTS test determines whether cells are metabolically viable. In both cases,

latex extracts (same extraction periods) were used as positive controls for cell death, while standard culture medium (formulation described below) was used as negative control for cell death.

2.7.1 Cell culture

The present experiment used a cell line of rat lung fibroblasts-L929, obtained from the European Collection of Cell Cultures (ECACC). Cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, USA), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% of an antibiotic–antimycotic mixture (Sigma, St. Louis, USA). For the MEM extraction tests, cells were seeded in 12 well plates ($n=3$, 1×10^5 cells/well), while for the MTS test cells were seeded in 96 well plates ($n=3$, 1.8×10^4 /well). In both cases cells were incubated for 24h at 37°C, in a 5% (v/v) CO₂ cell culture incubator.

2.7.2 MEM extraction test

Twenty four hours after cell seeding, culture medium was removed from the wells and an identical volume, 1 ml, of extraction fluid was added. Cultures were then incubated with the extracts for 72h, after which it was conducted a live/death assay by staining live cells with calcein-AM (1mg/ml; Molecular Probes, Oregon, USA) and non-viable cells with propidium iodide (PI, 0.1 mg/ml, Molecular Probes). Upon staining, cultures were observed under a fluorescence microscopy (Olympus BX-61, Hamburg, Germany).

2.7.3. *MTS test*

The metabolic viability of the L929 cells following exposure to extracts was determined by the bio-reduction of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS, Promega, USA), into a brown formazan product by dehydrogenase enzymes. The extraction procedure was performed as previously described, using, in this turn, 200 µl of extraction fluid per well [47]. After 72 h of incubation, extracts were removed and a mixture of DMEM with MTS (5:1 ratio) was added to each well. Cells were then incubated for 3 h at 37°C in a 5% CO₂ cell culture incubator after which O.D. was determined at 450 nm using a 96 well plate reader (Tecan Sunrise, Männedorf, Switzerland).

2.8. *Histocompatibility evaluation*

2.8.1. *Subcutaneous implantation*

Young male Wistar rats (8 weeks old) were purchased from Charles River, housed in light- and temperature-controlled rooms, and fed a standard diet. The maintenance and manipulation of animals were in accordance with standardized Animal Care Guidelines [48]. Hybrid 3D tubular scaffolds with one and two layers were implanted subcutaneously in the back of the animals for 7, 14, 21 and 28 days. At the end of the implantation period, rats were sacrificed. The scaffolds were removed from the animals, fixed in 3.7% formalin, embedded in Technovit 7100 (Kuzere, Germany) and processed for eosin-haematoxylin staining.

2.8.2. *Hemisection SCI model*

Young male Wistar rats (8 weeks old) were purchased from Charles River, housed in light- and temperature-controlled rooms, and fed a standard diet. The maintenance and

manipulation of animals were in accordance with standardized Animal Care Guidelines [48]. In order to expose the spinal cord a laminectomy was performed at T8-T8 level. Once exposed, a unilateral defect was performed on the spinal cord, by removing around 2-3mm of the latter. After this 3D semi-tubular scaffolds were implanted in the defect site, with the SPCL phase aligned with the vertebral while the gellan gum was facing, and filling, the injured spinal cord. The scaffolds were removed from the animals, fixed in 3.7% formalin, decalcified, paraffin embedded and finally processed for eosin-haematoxylin staining.

2.9. Encapsulation assays

For the cell encapsulation experiments within the gellan gum hydrogel, the human oligodendrocyte cell line MO3-13 was used. For this purpose, cells were grown as monolayer cultures in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic mixture until confluence. At this time cells were encapsulated in 2 different cellular densities (8 and 20×10^4 cells/ml) and injected into central canal of SPCL tubular scaffolds. Cell/Scaffolds constructs were then incubated in culture medium (DMEM, Gibco, 10% FBS, Gibco, 1% antibiotic/antimycotic, Sigma) for 7 days at 37°C in a 5% (v/v) CO_2 cell culture incubator. For this period, the distribution of viable cells was evaluated by confocal microscopy in combination with calcein-AM staining (Fluoview FV 1000, Olympus, Germany).

2.10. Statistical analysis

Statistical evaluation was performed using the ‘one way ANOVA’ test followed by the Tukey post-test, to assess the statistical differences between groups in the porosity and cytotoxicity analyses. Statistical differences evaluation on mechanical tests was

performed using a ‘two way ANOVA’ test followed by a Bonferroni post-test. Statistical significance was defined for $p < 0.05$.

3. Results and Discussion

The objective of this work was to develop a novel biphasic 3D tubular structure composed by a biodegradable semi-rigid polymer and a hydrogel-like material. Comparing with other currently used strategies for scaffold based SCI repair, our scaffold design has the advantages of combining the guidance stabilization from the 3D tubes, including the regeneration of vertebral bone, with the amenable conditions of hydrogel based material for cell and tissue migration. In this sense, our novel scaffold combines the beneficial effects of both strategies, overcoming the limitations of each strategy by itself. For instance, guidance tubes are able to stabilize the injured area from the mechanical point of view, while they act simultaneously as a physical guidance for SCI regeneration and protect the regenerating area from the invasion of adjacent connective tissue [2]. However, often this guidance tubes lack the signalling cues that allow a more efficient cell and tissue migration along the injured area [49]. Regarding hydrogels, it is known that they are quite versatile for SCI applications due to their cell encapsulation properties, peptide grafting and growth factor loading [28]. Nevertheless, in contrary to guidance tubes, hydrogels do not stabilize the injured area and have a limited capacity to avoid infiltration by the surrounding tissue [28]. The chosen biomaterials for the development of the 3D biphasic scaffolds have also several other advantages. Both of them are polysaccharides prone to normal degradation *in vivo* and both SPCL and gellan gum are easily integrated in different tissues. For instance, SPCL has been previously shown to allow the formation of bone extracellular matrix *in vitro*

and bone *in vivo* [39,41], which is one of the main objectives of the SPCL phase under the concept herein presented. Furthermore, this thermoplastic biomaterial is quite versatile from the processing point of view, allowing the use of rapid prototyping technologies for the development of the tubular structure. This is advantageous, as RP allows the processing of highly reproducible scaffolds. On the other hand, gellan gum rapidly polymerize either at room temperature or at 37°C which allows the encapsulation of relevant cell populations [50,51] without compromising their viability, as shown in the present manuscript (see below). Moreover, gellan gum is quite amenable for chemical modification, and thus it makes possible to graft and align relevant peptides that can promote axonal migration, such as RGD and IKVAV. In this context, standard aqueous-phase peptide synthesis methodologies will be used on the polysaccharide backbone to promote adequate binding of the sequences of interest to the gellan gum. This will be achieved by activating the carboxylic groups of the sugar molecules to which the pre-synthesized sequence of interest will be bound.

In order to develop the 3D guidance structure, a tubular scaffold was developed by combining rapid prototyping with a post-processing thermal treatment. As it can be observed in Figure 1, the proposed processing methodology was quite versatile allowing for the development of 3D tubular scaffolds with configurable outer layer thickness, fibre/filament orientation and pore geometry. Fibre orientation ranged from 0° to 90° and resulted from the design adopted during the rapid prototyping stage.

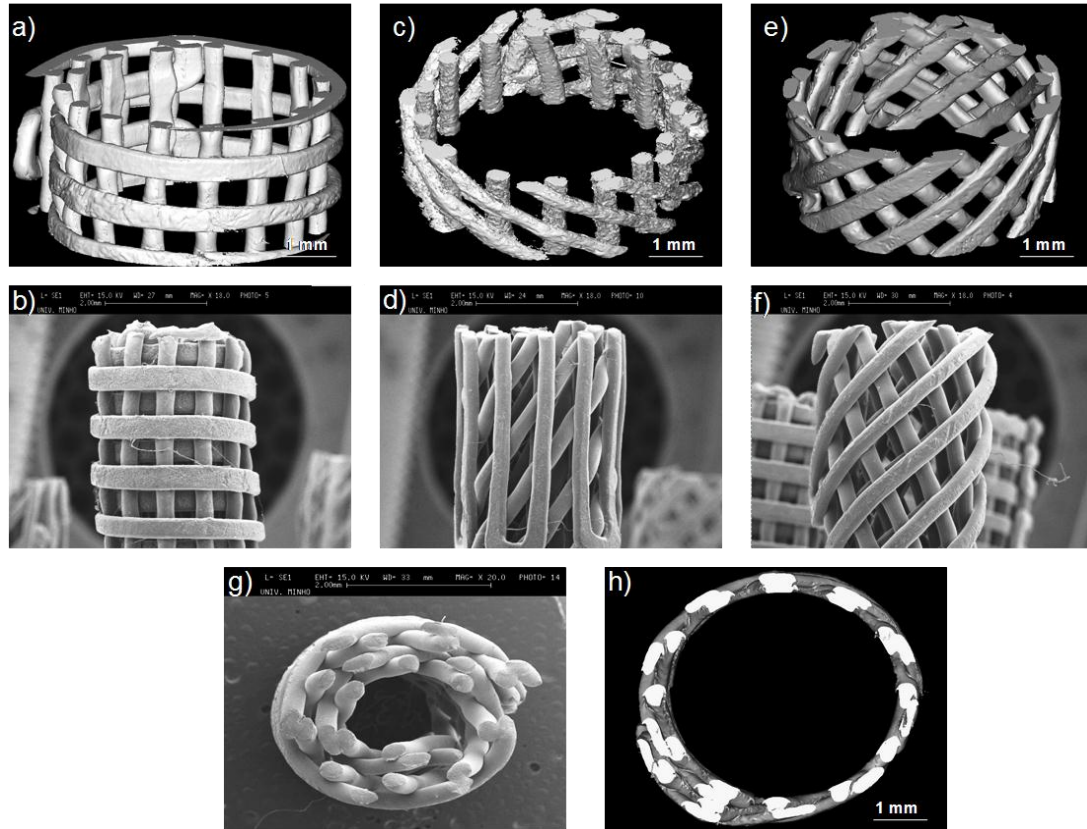


Figure III.1. SPCL tubular scaffolds, **a)** μ -CT image of 90/1 type structure; **b)** SEM of 90/2 type structure; **c)** μ -CT image of 45/1 type structure; **d)** SEM of 45/2 type structure; **e)** μ -CT image of X/1 type structure; **f)** SEM of X/2 type structure; **g)** μ -CT image of the top view of two layers type scaffolds; **h)** SEM of the top view of one layer type scaffolds.

Morphological analysis conducted by SEM and μ -CT demonstrated that the developed processing technique allowed the production of structures with a precise tubular geometry with fully interconnected pore networks. Further analysis conducted using μ -CT revealed that scaffolds had fully interconnected network of pores with approximately 85% porosity for structures featuring a single outer layer and around 71% porosity for structures featuring double outer layers (Figure 2). The differences in porosity observed were related to the outer thickness of the SPCL tubular structures, as the different orientations of the fibres did not affect the overall porosity of the scaffolds.

The porosity values are in the range of those found in the literature for similar purposes.

[52]

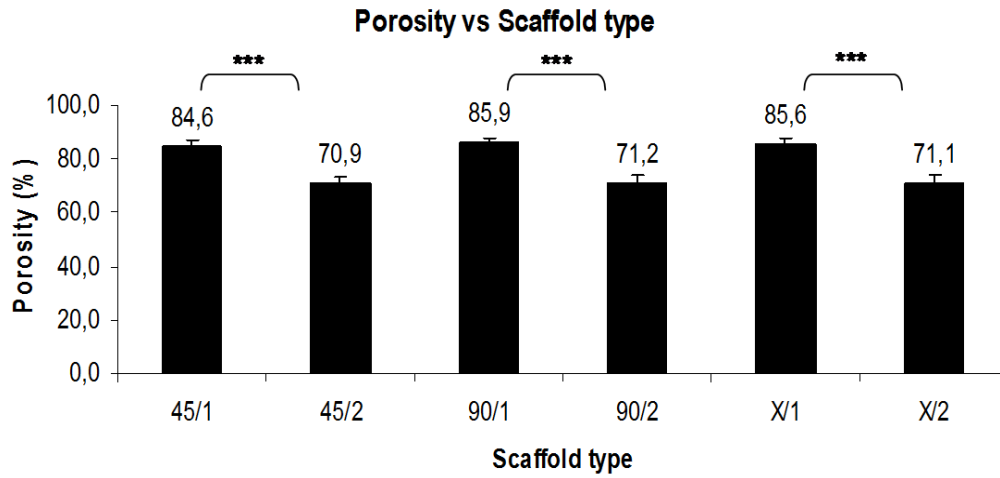


Figure III.2. Percentage values of porosity of each structure type. Mean \pm sd (n=3), *** statistical significance for $p < 0,001$.

In order to assess their mechanical properties, the different tubular scaffolds were subjected to dynamic compression testing. The objective of these experiments was to evaluate their mechanical integrity and simultaneously determine their respective stiffness. Tests were carried out, at 37°C in dry and wet conditions, in order to simulate and study the influence of physiologic conditions on the mechanical properties of the tubular scaffolds, namely the influence of water absorption. Moreover, to detect eventual mechanical collapse of the structure or layer detachment, the scaffolds were submitted to an increasing frequency test. Nevertheless, no major variation of storage modulus was detected upon the increase of frequency (Figure 3 a and b), which testifies the resilience of the scaffolds developed. Dry and wet tests revealed significant differences in the stiffness of the developed scaffolds. In the dry tests the 90/2 samples obtained the highest value, followed in decreasing order of magnitude by structures 45/2, 90/1, X/2, 45/1 and X/1 (Figure 3c, $p < 0,05$). Under wet conditions, as it would be

expected, there was a decrease in stiffness. Consistent with the dry test observations, conditions 90/2 and 45/2 had the highest values. However, in this case, the third condition that presented higher stiffness was X/2 followed by 90/1, 45/1 and X/1 (Figure 3d). The stiffness of the different tubular scaffolds at 1 Hz under dry and wet conditions is plotted in Figure 3e. The differences observed were related with different fibre orientations and scaffold thicknesses. Scaffolds with two layers had higher stiffness values as compared to the equivalent single layer structures. On what concerns to fibre orientation, scaffolds with aligned fibres along the tube axis had higher resistance to compression force. Our results revealed that, 90/1 and 90/2, as well as 45/1 and 45/2 conditions contained higher stiffness values when compared to those of X/1 and X/2 structures. For 90/1-2 and 45/1-2 scaffolds, half of the fibers were aligned in the same direction of the applied compression force. However, while for 90/1-2 scaffolds, approximately half of the fibres were aligned orthogonally to the compression force, for 45/1-2 conditions, the remaining half were diagonally aligned (45° relative to the compression force). In theory, 45/1-2 and X/1-2 conditions should present higher stiffness as compared to those experimentally observed, due to the stiffness contribution of diagonally orientated fibres - cosine (45°). The lower values of stiffness observed for scaffolds featuring diagonally orientated structures is probably due to buckling of the structure, which occurs upon compression due to geometrical instability of the structure. As previously shown (Figure 3e), under wet conditions, all scaffolds exhibited a decrease in stiffness, which can be explained by the water absorption. Water acts as plasticizer of the polymer, reducing its resistance to plastic deformation [53]. In SPCL, starch is the main water absorbent since PCL has low water uptake ability [53].

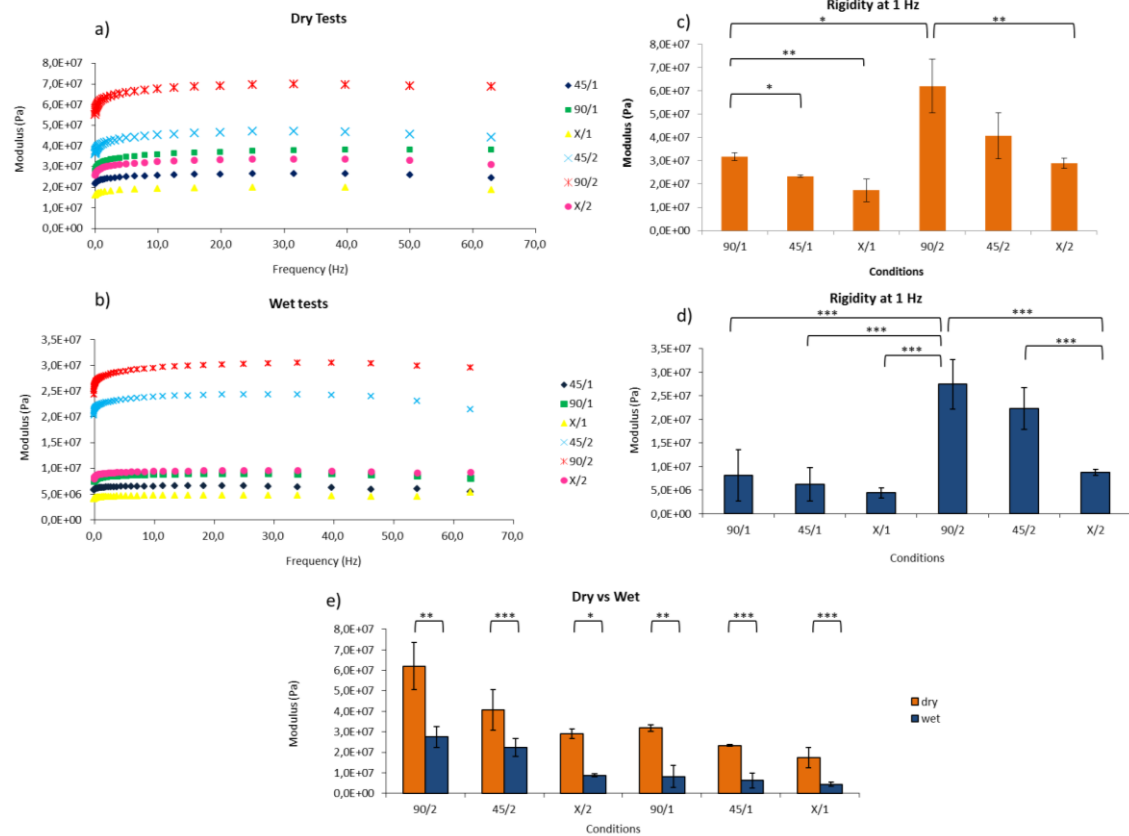


Figure III.3. Dynamic Mechanical Analyses of each scaffold type developed, **a)** Dry compressive modulus evaluation in function of frequency; **b)** Wet compressive modulus evaluation in function of frequency; **c)** Rigidity comparison at 1 Hz in dry conditions; **d)** Rigidity comparison at 1 Hz in wet conditions, **e)** Comparison at 1 Hz between the dry and wet tests. Mean \pm sd (n=3), *** Statistical significance for $p < 0,001$, ** for $p < 0,01$ and * for $p > 0,05$.

Furthermore, it could also be observed that the relative decrease in stiffness was larger in single layer structures. This can be explained by the larger surface area/total volume ratio of single layered structures as compared to double layered structures. In double layered scaffolds, the stacking of porous sheets reduces the relative surface area, which ultimately results in less water absorption. It is important to note that the mechanical tests with the SPCL structures were performed prior to gellan gum incorporation. Since the stiffness of gellan gum hydrogel is three orders of magnitude smaller than SPCL, no relevant mechanical effect would be expected for structures containing the hydrogel.

Under dry conditions, the elastic modulus for gellan gum was determined to be 79.7 ± 7.56 kPa, as characterized by Oliveira et al. [44].

Following the physical characterization of tubular scaffolds, the hydrogel phase was injected into the central canal of the tubes. This process allowed the formation of the biphasic structure presented in Figure 4. As it can be observe, the two phases were fully integrated in the system, being the central canal of the 3D SPCL tubular structure entirely filled with the gellan gum hydrogel. The main benefit of the proposed hybrid system arises from the individual potential advantages of each material. The SPCL frame is aimed at assuring the structural stiffness of the construct, while the gellan gum hydrogel is expected to create an amenable nerve regeneration environment.

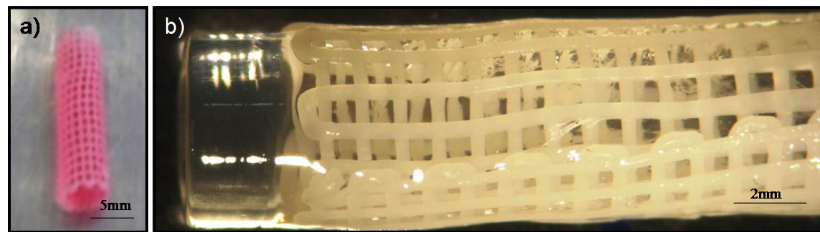


Figure III.4. First generation of FMSS, a) After maintain in culture with DMEM medium; b) FMSS loupe view (10x).

Biological assays were performed upon the development of the hybrid SPCL/gellan gum structures. Their cytotoxicity was determined by MTS and MEM extraction tests on a L929 cell line, while cell encapsulation assays were carried out with an oligodendrocyte-derived cell line. Since the SCPL layer will be used to connect to adjacent bone, the stiffness of the SPCL tubular structures is very important, as they will support directly the development of a mineralized tissue. In this sense, from all the developed structures, 90/2 samples were chosen for biological assays, as these present the best mechanical performance. Regarding cytotoxicity assays, MEM extraction tests

associated with calcein/PI staining revealed that the extracts obtained from the hybrid 3D tubular structures did not cause cell death among L929 cells. The cell density, morphology and viability were very similar between the extracted incubated cells and the cells grown in DMEM (positive control) (Figure 5a). These results were in contrast to cells grown in latex, and were then further confirmed by the MTS test (Figure 5b), under which L929 cells produced large amounts of a brown formazan product after incubation with the tested extracts. This is an indicator of normal metabolism as they were able to incorporate and metabolize MTS. MTS incorporation under these conditions was similar to the negative control. These results were supportive of a lack of cytotoxic effect of these hybrid structures.

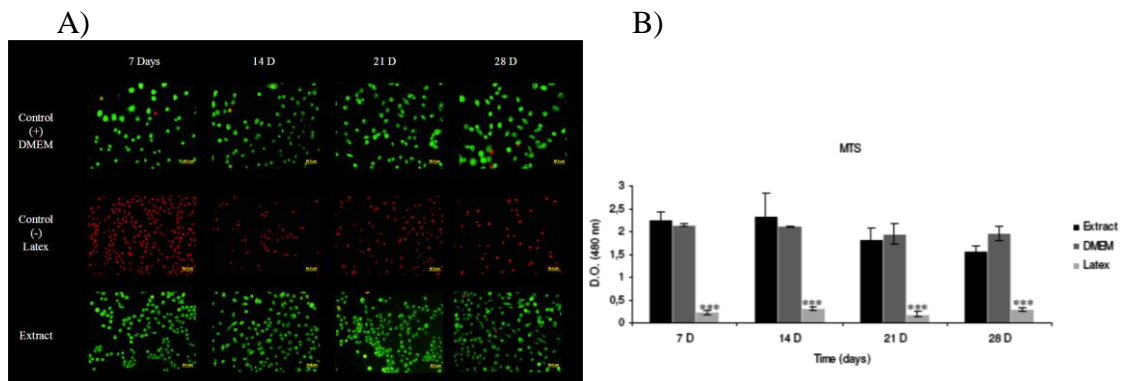


Figure III.5. Cytotoxicity assays. (A) MEM extraction evaluation of cell line L929 in four time-points of incubation (7, 14, 21 and 28 days) and three different conditions (positive control, negative control and FMSS extract). Viable cells were stained with calcein-AM and non-viable cells with PI. (B) MTS evaluation for cell line L929 in four time-points of incubation (7, 14, 21 and 28) and three different conditions (positive - DMEM - and negative - Latex - controls and FMSS extract). Mean \pm sd (n=3), *** Statistical significance for $p < 0,001$.

These favorable results were further confirmed by preliminary *in vivo* tests. In this case the 3D tubular hybrid structures were either subcutaneously implanted on Wistar rats (Figure 6a and 6b) or in a hemisection model of SCI (Figure 6c and 6d). In the subcutaneous model rats developed an initial, but mild, inflammatory reaction detected

after 1 week of implantation (Figure 6a), which faded by the fourth week (Figure 6b) indicating a possible biocompatible character of the scaffolds. Moreover, we also observed no formation of fibrotic capsules, supportive of the lack of inflammatory response triggered by the implanted scaffolds. Finally, it was possible to confirm that pore size, geometry and properties of the structure facilitated cells and connective tissue penetration within the scaffold. Similar results were also obtained for the SCI model, regarding the inflammatory response. As it can be seen in Figures 6 c) and d) no major inflammation processes were found. Moreover, it was possible to observe bone tissue (Figure 6c) forming around SPCL struts. Nevertheless, the results of this preliminary experiment will be further confirmed in future experiments.

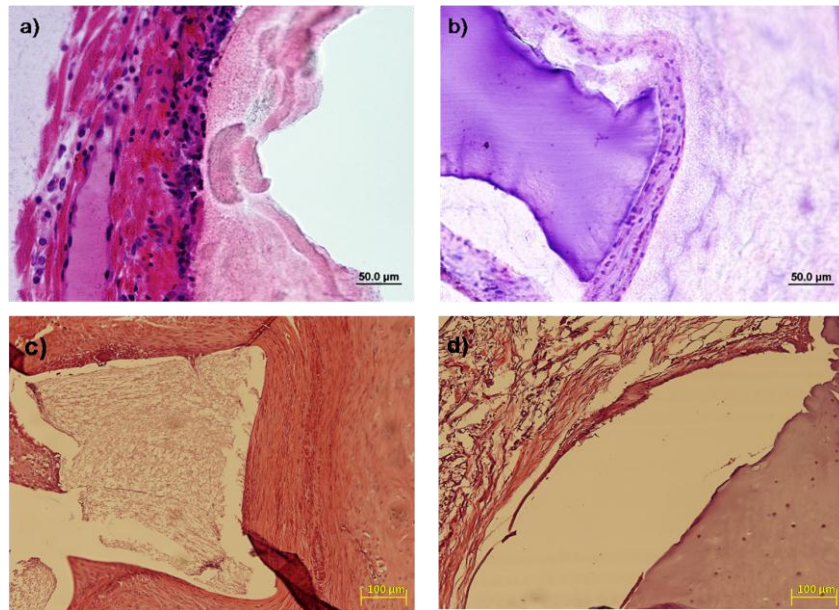


Figure III.6. Scaffolds were implanted subcutaneously (a, b) and in a hemisectioned spinal cord (c, d). In both cases histological sections were stained with hematoxylin–eosin. After 1 (a) and 4 (b) weeks of subcutaneous implantation, the tested hybrid scaffolds were well integrated within the surrounding tissue with minimal inflammatory response. Similar results were also obtained for the hemisection model (c, d), where it could be observed the formation of bone near SPCL struts.

The interaction of central nervous system cell populations with the developed structures is extremely important since they will be one of the mediators of nerve regeneration induction within the hydrogel phase. Therefore, preliminary cell encapsulations assays were performed using the MO3-13 human oligodendrocyte cell line. The rationale for using these cells was based on future approaches that can be used, namely the use of oligodendrocyte progenitors to foster remyelination repair in the affected SCI areas. As previously described the cell population was first encapsulated within the gellan gum hydrogel prior to injection in the tubular structure. Confocal laser microscopy observation revealed that the hydrogel phase of the hybrid structure supported the *in vitro* culture of MO3-13 cells during the culturing period (up to one week). The cells were preferentially dispersed within the gellan gum matrix, being either at the centre (Figure 7a) or at the vicinities (Figure 7b) of the hydrogel.

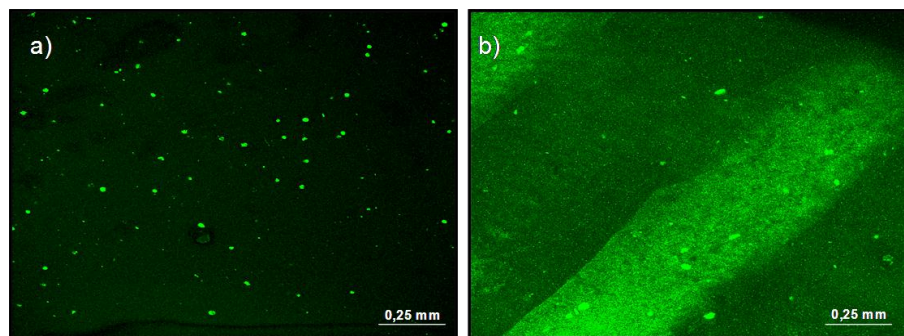


Figure III.7. Direct contact evaluation of cell line M0III encapsulation on FMSS. The green points are oligodendrocytes-like cells that appear dispersed on gellan gum matrix (a) and near the SPCL fibbers (b). Cells stained with calcein-AM.

4. Conclusions

With the present work it was possible to show that 3D plotting, a rapid prototyping technology was successfully used in the development of starch based scaffolds to be employed in future applications for SCI regeneration. We also showed that the

developed SPCL tubular scaffolds possessed configurable mechanical performance, pore sizes and interconnectivity values adequate for cell/tissue based regenerative applications. We also demonstrated that the different orientations of the fibres and the number of layers in the developed structures influenced their mechanical properties. Moreover, we demonstrated that SPCL/gellan gum hybrid structures were non-cytotoxic and disclosed an apparent biocompatible response *in vivo*. Finally, it was also possible to observe that these structures allowed the *in vitro* culture of oligodendrocyte like cells for periods up to one week. Further work is ongoing using these hybrid systems in animal models, in order to determine their potential to therapeutic intervention of SCI, as well the functionality of improved of gellan gum hydrogels in terms of cell adhesion and guidance upon division.

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References

- [1]. Sekhon LHS, and Fehlings MG., "Epidemiology, demographics, and pathophysiology of acute spinal cord injury", Spine 15, S2-S12, 2001.
- [2]. Samadikuchaksaraei A., "An overview of tissue engineering approaches for management of spinal cord injuries", J. NeuroEng and Rehab 14, 15, 2007.
- [3]. National Spinal Cord Injury Statistical Center: Spinal cord injury. Facts and figures at a glance. J Spinal Cord Med 28, 379, 2005.

- [4]. DeVivo MJ., "Causes and costs of spinal cord injury in the United States", *Spinal Cord* 35, 809, 1997.
- [5]. Ackery, A., Tator, C., Krassioukov, A., "A Global Perspective on Spinal Cord Injury Epidemiology", *Journal of Neurotrauma* 21, 1355, 2004.
- [6]. Winkler, T., Sharma, H.S., Gordh, T., Badgaiyan, R.D., Stalberg, E., Westman, J., "Topical application of dynorphin A (1-17) antiserum attenuates trauma induced alterations in spinal cord evoked potentials, microvascular permeability disturbances, edema formation and cell injury: an experimental study in the rat using electrophysiological and morphological approaches", *Amino Acids* 23, 273, 2002.
- [7]. Bao, F., John, S.M., Chen, Y., Mathison, R.D., Weaver, L.C., "The tripeptide phenylalanine-(D) glutamate-(D) glycine modulates leukocyte infiltration and oxidative damage in rat injured spinal cord", *Neuroscience* 140, 1011, 2006.
- [8]. Park, E., Velumian, A.A., Fehlings, M.G., "The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration", *Journal of Neurotrauma* 21, 754, 2004.
- [9]. Conti, A., Cardali, S., Genovese, T., Di Paola, R., La Rosa, G. "Role of inflammation in the secondary injury following experimental spinal cord trauma", *J Neurosurg Sci* 47, 89, 2003.
- [10]. Bracken, M.B., Shepard, M.J., Collins, W.F., Holford, T.R., Young, W., Baskin, D.S., Eisenberg, H.M., Flamm, E., Leo-Summers, L., Maroon, J., "A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury". Results of the Second National Acute Spinal Cord Injury Study, *The New England Journal of Medicine* 322, 1405, 1990.
- [11]. Bracken, M.B., "Methylprednisolone and acute spinal cord injury: an update of the randomized evidence", *Spine* 26, S47, 2001.
- [12]. Hurlbert, R.J., "Methylprednisolone for acute spinal cord injury: an inappropriate standard of care", *J. Neurosurg.* 93, 1, 2000.
- [13]. Bracken, M.B., Holford, T.R., "Neurological and functional status 1 year after acute spinal cord injury: estimates of functional recovery in National Acute Spinal Cord Injury Study II from results modeled in National Acute Spinal Cord Injury Study III", *J. Neurosurg.* 96, 259, 2002.
- [14]. Houweling, D.A., Bar, P.R., Gispén, W.H., Joosten, E.A., "Spinal cord injury: bridging the lesion and the role of neurotrophic factors in repair", *Prog. Brain Res.* 117, 445, 1998.

- [15]. Blesch, A., Lu, P., Tuszynski, M.H., "Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair", *Brain Res. Bull.* 57, 833, 2002.
- [16]. Jones, L.L., Oudega, M., Bunge, M.B., Tuszynski, M.H., "Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury" *J. Physiol.* 533, 83, 2001.
- [17]. David, S., Lacroix, S., "Molecular Approaches to Spinal Cord Repair", *Annual Review of Neuroscience* 26, 411, 2003.
- [18]. Chau, C.H., Shum, D.K., Li, H., Pei, J., Lui, Y.Y., Wirthlin, L., Chan, Y.S., Xu, X.M., "Chondroitinase ABC enhances axonal regrowth through Schwann cell-seeded guidance channels after spinal cord injury", *FASEB J.* 18, 194, 2004.
- [19]. Wunderlich, G., Stichel, C.C., Schroeder, W.O., Müller, H.W., "Transplants of immature astrocytes promote axonal regeneration in the adult rat brain", *Glia* 10, 49, 1994.
- [20]. Hikawa, N., Horie, H., Takenaka, T., "Macrophage-enhanced neurite regeneration of adult dorsal root ganglia neurones in culture", *Neuroreport* 5, 41, 1993.
- [21]. Ramón-Cueto, A., Cordero, M.I., Santos-Benito, F.F., Avila, J., "Functional Recovery of Paraplegic Rats and Motor Axon Regeneration in Their Spinal Cords by Olfactory Ensheathing Glia", *Neuron* 25, 425, 2000.
- [22]. Myckatyn, T.M., Mackinnon, S.E, McDonald, J.W., "Stem cell transplantation and other novel techniques for promoting recovery from spinal cord injury", *Transplant Immunology* 12, 343, 2004.
- [23]. Kakulas, B.A., "Neuropathology: the foundation for new treatments in spinal cord injury", *Spinal Cord* 42, 549, 2004.
- [24]. Syková, E., Jendelová, P., Urdzík, L., Lesný, P., Hejčl, A., "Bone Marrow Stem Cells and Polymer Hydrogels - Two Strategies for Spinal Cord Injury Repair" *Cell Mol Neurobiol.* 26, 1111, 2006.
- [25]. Woerly, S., Doana, van D., Sosa, N., Vellis, J., Espinosa, A., "Reconstruction of the transected cat spinal cord following NeuroGel™ implantation: axonal tracing, immunohistochemical and ultrastructural studies", *International Journal of Developmental Neuroscience* 19, 63, 2001.
- [26]. Hejčl, A., Lesný, P., Přádný, M., Michálek, J., Jendelová, P., Štulík, J., Syková, E., "Biocompatible hydrogels in spinal cord injury repair", *Physiol Res.* 2008. *in press*
- [27]. Tsai, E.C., Dalton, P.D., Shoichet, M.S., Tator, C.H., "Synthetic hydrogel guidance channels facilitate regeneration of adult rat brainstem motor axons after complete spinal cord transaction", *J. Neurotrauma* 21, 789, 2004.

- [28]. Salgado, A.J., Silva, N.A., Neves, N.M., Reis, R.L., Sousa, N., "Hydrogel Based Systems for Spinal Cord Injury Regeneration", Reis RL ed., In *Natural-based Polymers for Biomedical Applications*, Cambridge, Woodhead Publishing Limited, 2008.
- [29]. Langer, R., Vacanti, J.P., "Tissue engineering", *Science* 260, 920, 1993.
- [30]. Liu, S., Said, G., Tadie, M., "Regrowth of the rostral spinal axons into the caudal ventral roots through a collagen tube implanted into hemisected adult rat spinal cord", *Neurosurgery* 49, 143, 2001.
- [31]. Yoshii, S., Ito, S., Shima, M., Taniguchi, A., Akagi, M., "Functional restoration of rabbit spinal cord using collagen-filament scaffold", *J Tissue Eng Regen Med* 3, 19, 2009.
- [32]. Ying, L., Shoichet, M., "Light-Activated Immobilization of Biomolecules to Agarose Hydrogels for Controlled Cellular Response", *Biomacromolecules* 5, 2315, 2004.
- [33]. King, V.R., Henseler, M., Brown, R.A., Priestley, J.V., "Mats made from fibronectin support oriented growth of axons in the damaged spinal cord of the adult rat", *Exp Neurol* 182, 383, 2003.
- [34]. Taylor, S.J., McDonald III J.W., Sakiyama-Elbert S.E., (2004), "Controlled release of neurotrophin-3 from fibrin gels for spinal cord injury", *J Controll Release* 98, 281, 2004.
- [35]. Reis, R.L., Cunha, A.M., "Characterization of two biodegradable polymers of potential application within the biomaterials field", *J. Mater. Sci. Mater. Med.* 6, 786, 1995.
- [36]. Cascone, M.G., Barbani, N., Cristallini Giusti, C., Ciardelli, G., Lazzeri, L., "Bioartificial polymeric materials based on polysaccharides", *J. Biomat. Sci. Polym. Ed.* 12, 267, 2001.
- [37]. Reis, R.L., Mendes, S.C., Cunha, A.M., Bevis, M.J., "Processing and in vitro degradation of starch/EVOH thermoplastic blends", *Polym. Intern.* 43, 347, 1997.
- [38]. Malafaya, P.B., Elvira, C., Gallardo, A., San Román, J., Reis, R.L., "Porous starch-based drug delivery systems processed by a microwave route", *J. Biomed. Sci. Polym. Edn.* 12, 1227, 2001.
- [39]. Gomes, M.E., Sikavitsas, V.I., Behraves, E., Reis, R.L., Mikos, A.G., "Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds" *Tissue Eng.* 12(4): 801, 2006.

- [40]. Reis, R. L., Cunha, A. M., "Starch Polymers", Encyclopedia of Materials: Science and Technology, Elsevier Science Ltd, 2001, pp. 8810-8816.
- [41]. Mendes, S.C., Bezemer, J., Claase, M.B., Grijpma, D.W., Bellia, G., Degli-Innocenti, F., Reis, R.L., de Groot, K., van Blitterswijk, C.A., de Bruijn, J.D., "Evaluation of two biodegradable polymeric systems as substrates for bone tissue engineering.", Tissue Eng. Suppl 1:S91, 2003.
- [42]. Martins, A., Chung, S., Pedro, A.J., Sousa, R.A., Marques, A.P., Reis, R.L., Neves, N.M., "Hierarchical starch-based fibrous scaffold for bone tissue engineering applications", J Tissue Eng Regen Med 3, 37, 2009.
- [43]. Salgado AJ , Sousa RA, Fraga JS, Pego JM, Silva BA, Malva JO, Neves NM, Reis RL and Sousa N, 2007, Preliminary Study on the Effects of Starch/Polycaprolactone Based Blends Aimed to be Used for Spinal Cord Injury Regeneration in Hippocampal Neurons/Glial Cells Viability and Proliferation, Journal of Bioactive and Compatible Polymers. submitted
- [44]. Oliveira, J.T., Costa-Pinto, A.R., Martins, L., Malafaya, P.B., Sousa, R.A., Mano, J.F., Neves, N.M., Reis, R.L., "Gellan Gum Hydrogels as Supports for Human Articular Chondrocytes and Human Bone Marrow Cells for Cartilage Tissue Engineering Application", Abstract presented at ESF/EMBO Symposium Stem Cells in Tissue engineering isolation, culture, characterisation and applications, Sant Feliu de Guixols, Spain, 2006.
- [45]. Yeong, W.Y., Chua, C.K., Leong, K.F., Chandrasekaran, M., "Rapid prototyping in tissue engineering: challenges and potential", Trends in Biotechnology 22, 643, 2004.
- [46]. Leong, K.F., Cheah, C.M., Chua, C.K., "Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs", Biomaterials 24, 2363, 2003.
- [47]. Salgado, A.J., Coutinho, O.P., Reis, R.L., "Novel Starch Based Scaffolds for Bone Tissue Engineering: Cytotoxicity, Cell Culture and Protein Expression", Tissue Engineering 10, 665, 2004.
- [48]. Van Zutpen, LFM., Baumans, V., Beynen, AC. Principles of Laboratory Animal Science. Amsterdam: Elsevier, 2001.
- [49]. Kerschensteiner, M., Schwab, M. E., Lichtman, J. W., Misgeld, T., "In vivo imaging of axonal degeneration and regeneration in the injured spinal cord", Nature Medicine 11, 572, 2005.

- [50]. Oliveira, J.T., Gardel, L., Rada, T., Martins, L., Gomes, M.E., Reis, R.L., "Rabbit Articular Cartilage Full-Thickness Size Defects Treated With Novel Gellan Gum Injectable Hydrogels and Autologous Adipose Stem Cells", *Tissue Engineering part A*, 2008 (submitted).
- [51]. Oliveira, J.T., Santos, T.C., Martins, L., Silva, M.A., Marques, A.P., Castro, A.G., Neves, N.M., Reis, R.L., "Performance of New Gellan Gum Hydrogels Combined With Human Articular Chondrocytes for Cartilage Regeneration When Subcutaneously Implanted In Nude Mice", *Journal of Tissue Engineering and Regenerative Medicine (TERM)*, 2008, (submitted).
- [52]. Moore, M.J., Friedman, J.A., Lewellyn, E.B., Mantila, S.M., Krych, A.J., Ameenuddin, S., Knight, A.M., Lu, L., Currier, B.L., Spinner, R.J., Marsh, R.W., Windebank, A.J., Yaszemski, M.J., "Multiple-channel scaffolds to promote spinal cord axon regeneration", *Biomaterials* 27, 419, 2006.
- [53]. Alves, N.M., Saiz-Arroyo, C., Rodriguez-Perez, M.A., Reis, R.L., Mano, J.F., "Microhardness of starch based biomaterials in simulated physiological conditions", *Acta Biomater.* 3, 69, 2007.

CHAPTER IV

Benefits of Spine Stabilization with Biodegradable Scaffolds in Spinal

Cord Injured Rats

Chapter IV.

Benefits of Spine Stabilization with Biodegradable Scaffolds in Spinal Cord Injured Rats*

Abstract

Spine stabilization upon spinal cord injury (SCI) is a standard procedure in clinical practice, but rarely employed in experimental models. Moreover, the application of biodegradable biomaterials for this would come as an advantage as it would eliminate the presence of a non-degradable prosthesis within the vertebral bone. Therefore, in the present work, we propose the use of a new biodegradable device specifically developed for spine stabilization in a rat model of SCI. A 3D scaffold based on a blend of starch with polycaprolactone (SPCL) was implanted, replacing delaminated vertebra, in male Wistar rats with a T8-T9 spinal hemisection. The impact of spinal stabilization on the locomotor behavior was then evaluated for a period of 12 weeks. Locomotor evaluation, assessed by Basso, Beattie and Bresnahan (BBB) test, rotarod and open field analysis, revealed that injured rats subjected to spine stabilization significantly improved their motor performance, including higher coordination and rearing activity when compared to SCI rats without stabilization. Histological analysis further revealed that the presence of the scaffolds not only stabilized the area, but simultaneously prevented the infiltration of the injury site by connective tissue. Overall, these results reveal that SCI stabilization using a biodegradable scaffold at the vertebral bone level, leads to an improvement of the motor deficits and is a relevant element for the successful treatment of SCI.

***These chapter is based on the following publication:**

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1. Introduction

Spinal cord injury (SCI) represents a significant health and social problem. It was estimated that approximately 500,000 people, in the US and Europe alone, have to deal each day with the burden of having a spinal cord injury (SCI). Importantly, the incidence of SCI lies between 10.4 and 83 per million inhabitants each year, in western countries [1, 2].

Currently, medical practice for SCI patients is mainly based on 3 steps: stabilization of the spine, using metallic/vertebral spinal fusions [3]; decompression of the cord [4]; and administration of the anti-inflammatory drug methylprednisolone (MP) [5]. Regarding spine stabilization, there is presently an open debate among physicians concerning the timing of surgery, even though there is strong evidence within the literature that early surgical stabilization consistently leads to shorter hospital stays, shorter intensive care unit stays, less days on mechanical ventilation, and less pulmonary complications [3]. Nevertheless, the use of metallic devices, such as spinal fusions, have some disadvantages, namely the potential need of a second surgery to remove it as well as interfering with magnetic resonance imaging during postoperative follow-up [6]. Dynamic stabilization is a promising alternative to traditional spinal fusions, Cakir and colleagues [7], for instance, presented favorable short-term results when this system was applied. However, the longevity of a dynamic stabilization construct in an active adult, with constant motion, is an important consideration. Some devices have been abandoned because of failure over time [8]. Moreover, Goldstein and colleagues demonstrated that the infection rate in patients undergoing dynamic stabilization is higher than that for instrumented fusion [9]. We believe that biocompatible scaffolds that can act as stabilization devices and at same time promote bone regeneration are a very promising alternative to both traditional and dynamic devices. Biodegradable tools

have been studied, namely in the form of 3D scaffolds. However, to date, none of this have been specifically designed for spine stabilization after SCI [6]. Surprisingly, in rat SCI models, spine stabilization is not performed at all. However, the majority of the surgical procedures used in rat SCI models comprise a laminectomy which may affect the locomotor behavior and trunk stability of the animals. In this sense, the absence of spine stabilization may compromise the regenerative process of the spinal cord tissue after the injury.

In order to specifically target this problem we previously reported the development and characterization of a biodegradable scaffold composed of a blend of starch with polycaprolactone (SPCL) aimed for spine stabilization. SPCL scaffolds were fabricated by rapid prototyping and have shown to disclose appropriate mechanical performance, *in vitro* non-cytotoxic behavior and *in vivo* biocompatibility [10]. Moreover, SPCL has been originally proposed by our group for a wide range of tissue engineering applications including bone regeneration [11-13]. In this sense, the objective of the present study was to evaluate to what extent the stabilization of the vertebral column through the implantation of the referred scaffold is feasible and whether it promotes beneficial motor effects.

2. Materials & Methods

2.1. Processing of SPCL scaffolds.

The SPCL scaffolds were processed as previously reported [10]. Briefly, porous sheets featuring inter filament orientations of 90° were produced by 3D plotting, a rapid prototyping technology (Bioplotter®, Envisiontec GmbH, Germany). Tubular scaffolds were obtained by rolling up porous sheets around a cylinder and subsequent heat

treatment at 65° C during 30 min for inducing the adhesion between filaments. Scaffolds were then cut in semi-tubular structures (Fig. 1a and b). All the materials were sterilized with ethylene oxide.

2.2. Animals

Eight week old male Wistar rats (Charles River, Barcelona), housed in light and temperature controlled rooms and fed with standard diet, were used in this study. The Animal Care Committee of the Research Institute approved the animal protocols in accordance with standardized Animal Care Guidelines [14]. After SCI, the animals were divided into 2 experimental groups: animals with spine stabilization through SPCL scaffolds implantation (SPCL, n=9) and animals without spine stabilization (SCI, n=5). A third group of animals, subjected to laminectomy only, were used as controls (Sham, n=5). Handling was performed for 3 days before the surgery.

2.3. Surgery and post-operative care

All animals were anesthetized by intraperitoneal injection of a mixture (1,5:1) of ketamine (60 mg/ml, Imalgen, Merial, Portugal) and medetomidine hydrochloride (0.4 mg/ml, Dorbene Vet, Laboratorios SYVA S.A., Spain). Once anaesthetized, fur was shaved from the surgical site and the skin disinfected with chlorohexidine (AGB, Spain). Then a dorsal midline incision was made from T6–T11 and the paravertebral muscles retracted. A laminectomy was performed at the junction T8–T9 in which the spinous processes were removed and the spinal cord exposed. Two hemisections were performed, 3 mm apart on the left side, and the tissue in between removed. SPCL

scaffolds were implanted at the vertebral bone level, juxtaposed to the spinal cord, providing spine stabilization (Fig 1c). Bone cement (Biomet, USA) was used to fix the scaffolds margins to bone. Paravertebral muscles and skin were then separately closed with Vicryl sutures (Johnson and Johnson, USA). The incision of control animals was closed after SCI, without SPCL implantation. Following surgery rats were kept under heat lamps and received vitamins (diphalyte, Farmoquil, Portugal), analgesic (butorphanol tartrate, 1mg/ml, Fort Dodge, Spain) and antibiotic (enrofloxacin, 1mg/ml, Bayer, Germany). Bladder evacuation was done manually. Throughout the treatment and recovery period, animals were examined for symptoms of illness or potential reaction to the treatment.

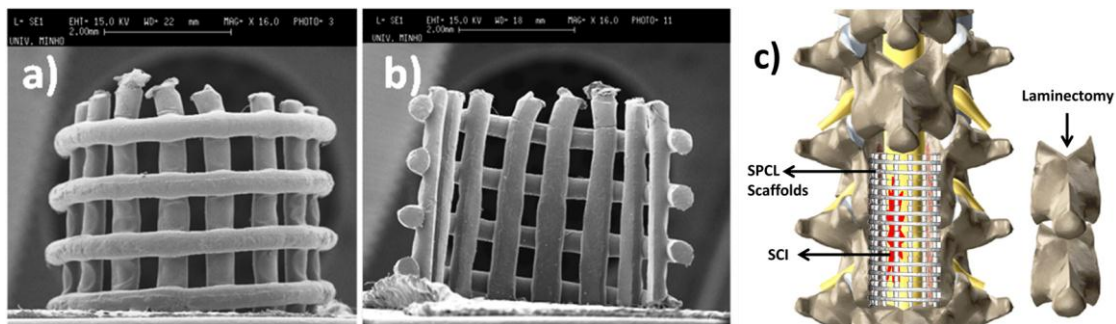


Figure IV.1. Scanning electron microscope pictures of SPCL scaffolds used for spine stabilization (a,b). Schematic representation of spine stabilization using SPCL scaffolds (c). The biodegradable scaffolds were implanted at the vertebra level using bone cement.

2.4. Assessment of locomotor function by BBB test

All rats were assessed with the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) [15] on day 3 and 2, 5, 7, 9, 12 weeks after injury. The BBB is a 21-point scale designed to assess hindlimb locomotor recovery following thoracic spinal cord injury. A

BBB score of 0 indicates no hindlimb movement. A BBB score of 1 through 8 indicates joint movement, but no weight support. A BBB score of 9 through 20 indicates an ability to support weight and use the limb for locomotion but with some degree of abnormality. A BBB score of 21 corresponds to the locomotion of a normal rat.

2.5. Motor behavior analyses in an open field chamber

The open field is a versatile test that permits the assessment of motor behavior by measuring the amount of rearing activity and the total distance travelled by the rats [16]. The OF was performed in a square (43.2 cm \times 43.2 cm) arena with transparent acrylic walls (Med Associates Inc., St. Albans, VT, USA) placed in a brightly illuminated room. Animals started the test at the arena's center and were given 5 min to explore it. Total distance travelled in the arena and number of rearings was automatically registered by equipment sensors.

2.6. Rotarod test

Motor coordination of the animals was evaluated in a rotarod equipment (TSE systems, Germany). Each animal was placed on a 10-cm diameter, 15-cm long rod, rotating at constant speed. Impairment of motor coordination was defined as the inability of rats to remain on the rotating rod for a 60s test period. Experimentally animals were pre-trained on the rotating track 24 h before the proper test. The protocol consisted of 3 days of testing at 4, 8 and 12 rpm in, respectively, 1st, 2nd and 3rd day for a maximum of 60s in four trials, with a 10 min interval between each trial. The latency to fall (in seconds) was recorded by equipment sensors.

2.7. *Tissue preparation*

Twelve weeks after the scaffold implantation, the rats were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (Ceva Saude Animal, Portugal). Then, animals were perfused through the ascending aorta with 4% paraformaldehyde in PBS. A 2.5-3 cm length of spine and spinal cord, centered on the site of hemisection and scaffold placement, was carefully removed and fixed in neutral buffered formalin. After decalcification, spinal cord and spine were carefully split and then embedded in paraffin and processed for hematoxylin–eosin staining. The tissue was sectioned on the coronal plane and apoptotic cells, vacuolated neurons and tissue infiltration/organization was evaluated on three tissue slices of each subdivision (dorsal, median and ventral cord). Apoptotic cells and vacuolated neurons were counted both rostral and caudal to the injury center and on the left side of the cord.

2.8. *Statistical analysis*

To assess if the values come from a Gaussian distribution the BBB, Open Field, Rotarod, and histological data was analyzed using the Kolmogorov-Smirnov test. Then, to evaluate statistical differences among groups a ‘two way ANOVA’ test followed by a Bonferroni post-test was performed. Statistical significance was defined for $p < 0.05$. All data are presented as mean \pm SEM.

3. Results

3.1. *Locomotor Function Evaluation by BBB test*

Left hindlimb function was evaluated one day after the hemisection injury. Only the animals that were completely paralyzed were selected for the study. From the initial 24 animals subjected to surgery, two died during the surgical procedure (from groups sham and SCI) and another two died during the experimental protocol (from groups SCI and SPCL). Motor evaluation began 3 days after surgery and continued each 2-3 weeks for 3 months. In all experiments the identity of the animals was kept blind to the observer. After SCI, SPCL scaffolds were implanted in the vertebral column of 9 animals (Fig. 1). Five weeks after, animals stabilized with SCPL scaffolds presented significant motor improvements when compared to the non-stabilized group (Fig. 2). Moreover, these differences persisted up to 12 weeks. Injured animals subjected to spine stabilization presented, extensive movements of all three joints of the left hindlimb (SPCL, 7.3 ± 1.5 in the BBB score), whereas rats without spine stabilization presented slight to extensive movements of just one joint (SCI, 1.8 ± 1.1 in the BBB score) (Fig. 2). Animals from sham group scored 21 (maximum) in BBB scale, showing that laminectomy alone did not affect the animals motor skills (Fig. 2).

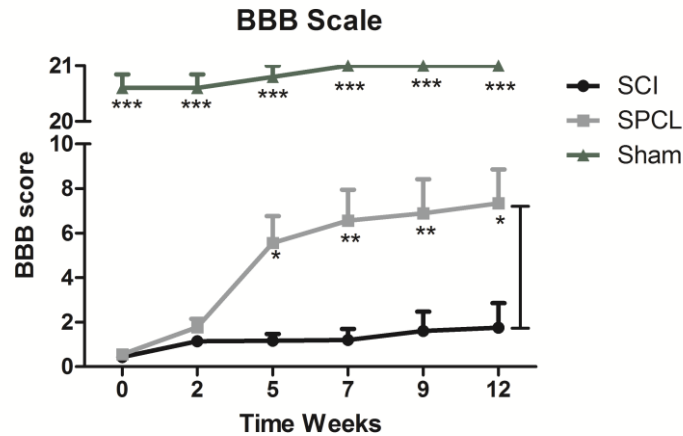


Figure IV.2. BBB locomotor scale evaluation of SCI rats with spine stabilization (SPCL, n=9), without spine stabilization (SCI, n=5) and animals subjected only to a laminectomy (Sham, n=5). The BBB test showed significant motor skill improvement in rats with spine stabilization ($p<0.05$), comparing to those without stabilization. Sham animals did not present motor impairments ($p<0.001$). Values are shown as mean \pm SEM, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

3.2. Open Field Analyses

Unlike the BBB, the Open Field (OF) test is dependent on animal motivation to explore a new environment. Since the repetition of the same environment could lead to a decrease of the exploratory behavior, the OF test was not performed each 2 weeks. Instead, OF analyses were carried out in the first and last weeks of the experimental protocol and in different rooms. The total distance traveled in the OF arena just after the surgery was not significantly different between groups (266.6 ± 19.3 cm for SPCL and 361.5 ± 99.0 cm for SCI). However, at 12 weeks the total distance travel by animals with a spine stabilized was significantly greater than that seen in the non-stabilized animals (1272.6 ± 184.4 cm for SPCL and 673.5 ± 161.0 cm for SCI) (Fig.3a). Moreover, the number of rearings (exploratory behavior where animals stand up only in their hindpaws) was also assessed. Once again animals from both groups had a similar performance after surgery (5.4 ± 1.7 for SPCL 4.3 ± 1.8 and for SCI) while at 12 weeks,

animals with SPCL implantation performed significantly more rearing activity than non-stabilized animals (25.7 ± 3.8 for SPCL and 12.5 ± 2.8 for SCI) (Fig.3b). Importantly, despite a remarkable difference to sham animals in distance and rearings after surgery, 12 weeks after stabilization, animals did not show statistical differences (Fig.3).

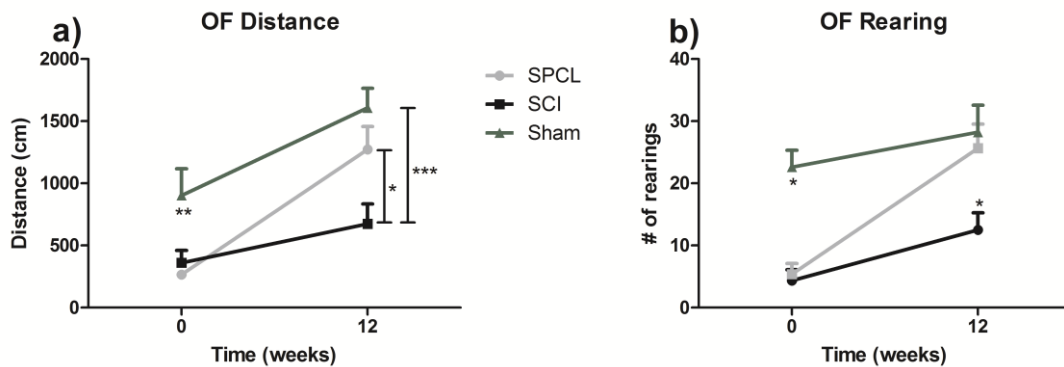


Figure IV.3. Distance and rearing behavior evaluation. When tested in an open field apparatus, injured rats with spine stabilization (SPCL) performed significantly better ($p < 0.05$), both in distance traveled (a) and in number of rearings (b), comparing to non-stabilized animals (SCI). Comparing to the sham group, spine stabilized animals (SPCL) performed worse in the first week ($p < 0.05$) however at 12 weeks both groups performed equally, showing motor improvements. Values are shown as mean \pm SEM, * $p < 0.05$; *** $p < 0.001$.

In addition to the above results, we also performed the open field test in animals that did not suffer any surgery which was used as baseline. Interestingly, these animals performed significantly more rearings ($p < 0.01$) than the sham animals but covered approximately the same distance (Fig.4). This data indicates that, although the hindlimb function of the sham animals was not affected (distance covered), they presented specific difficulties on rearing behavior. This difference on rearing behavior is most likely due to the absence of spine stabilization on sham animals.

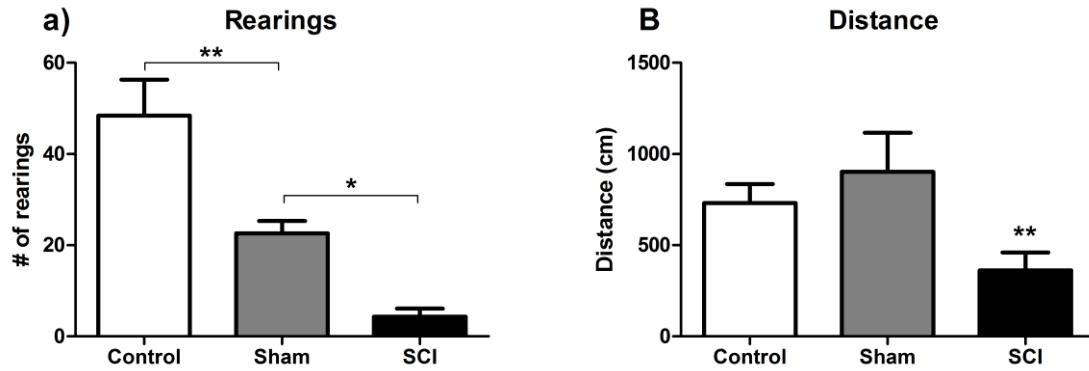


Figure IV.4. Open field analysis one week upon surgery. When tested in an open field apparatus, rats with a laminectomy (sham) performed significantly less rearing than control animals (rats without any surgery) (**a**). Both control and sham animals covered the approximately the same distance (**b**) and SCI animals presented significantly less distance covered and rearings. Values are shown as mean \pm SEM, * $p < 0.05$; ** $p < 0.01$.

3.3. RotaRod Test

To assess motor coordination, a rotarod test was performed in the last week of the experiment. Once again, this test was not performed throughout all the experimental protocol because the falls from the rotating rod could lead to further injuries. The test was performed on 3 consecutive days. In first, at 4 rpm, spine stabilized animals latency to fall from the rotating rod was significantly higher than non-stabilized animals (27.1 ± 8.6 s for SPCL and 2.2 ± 1.2 s for SCI). However, at 8 and 12 rpm (respectively day 2 and 3) both stabilized and non-stabilized animals showed lack of motor coordination. The latency to fall happened only a few seconds from the start of the trial (8rpm, 4.1 ± 1.1 s; 12rpm, 4.9 ± 1.8 s for SPCL and 1.8 ± 1.9 ; 2.7 ± 1.6 for SCI). Once again, sham animals successfully executed the test, showing that their motor coordination was not affected by the laminectomy (Fig. 5).

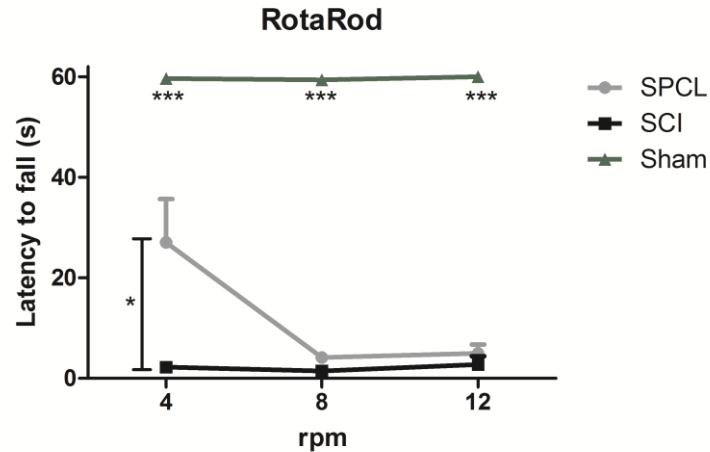


Figure IV.5. Motor coordination assessment. In the Rotarod test, rats with stabilization presented significantly higher motor coordination at 4rpm ($p<0.05$), however, for more demanding task (8rpm and 12 rpm), rats performed equal to non-stabilized animals. Sham animals successfully performed the test, showing no motor coordination impairment. Values are shown as mean \pm SEM, * $p<0.05$; *** $p<0.001$.

3.4. Histological Characterization

Hematoxylin and eosin (H&E) staining was performed to assess the effects of spine stabilization on the spinal cord organization, surrounding tissue infiltration and axon vacuolization after a SCI. A decrease of connective tissue infiltration into the spinal cord after the injury in animals subjected to spine stabilization was observed (Fig. 6a and b). As shown in figure 6a, animals without scaffold implantation have a higher connective tissue infiltration. Less connective tissue infiltration was observed in treated animals after injury (Fig.6b). When observed at higher magnification it was possible to detect less cell death and less vacuolated neurons in injury animals with spine stabilization then in non-stabilized animals (Fig. 6d, e, g and h). We observed that the number of apoptotic cells was significantly different between groups. There was a 4.9

fold increase (ratio to intact spinal cord) in the number of apoptotic cells found in the spinal cord tissue of non-stabilized animals and only a 2.0 fold increase (ratio to sham spinal cord) in the spinal cord of animals with spine stabilization (Fig. 6g). A similar trend was observed in the number of vacuolated neurons. Animals without spine stabilization presented significantly more vacuolated neurons (10.1 fold increase) than stabilized animals (3.6 fold increase; Fig. 6h). The histological analysis of the vertebral bone revealed that SPCL scaffolds were able to support new bone formation. The typical morphology of newly formed bone is characterized by a high number of cells and extracellular matrix more intensely stained for hematoxylin than eosin [17]. Both of these features were found in the tissue between the SPCL fibers (Fig.6f). Moreover, it was also possible to observe a few attributes typical in mature bone, such as some possible lamellar organization as well as spaces within the bone matrix, known as *lacunae*, which contain osteocytes. These results revealed that newly formed bone involving the SPCL fibers started maturation. This is contrary to what is seen in animals that did not receive SPCL implants. In those animals we only observe the formation of granulation tissue and apparently no bone formation yet (Fig. 6i). The granulation tissue (mainly formed by fibroblast and periosteal cells) is a standard response after bone fractures. This new loose connective tissue provides a temporary extracellular matrix that supports new bone formation.

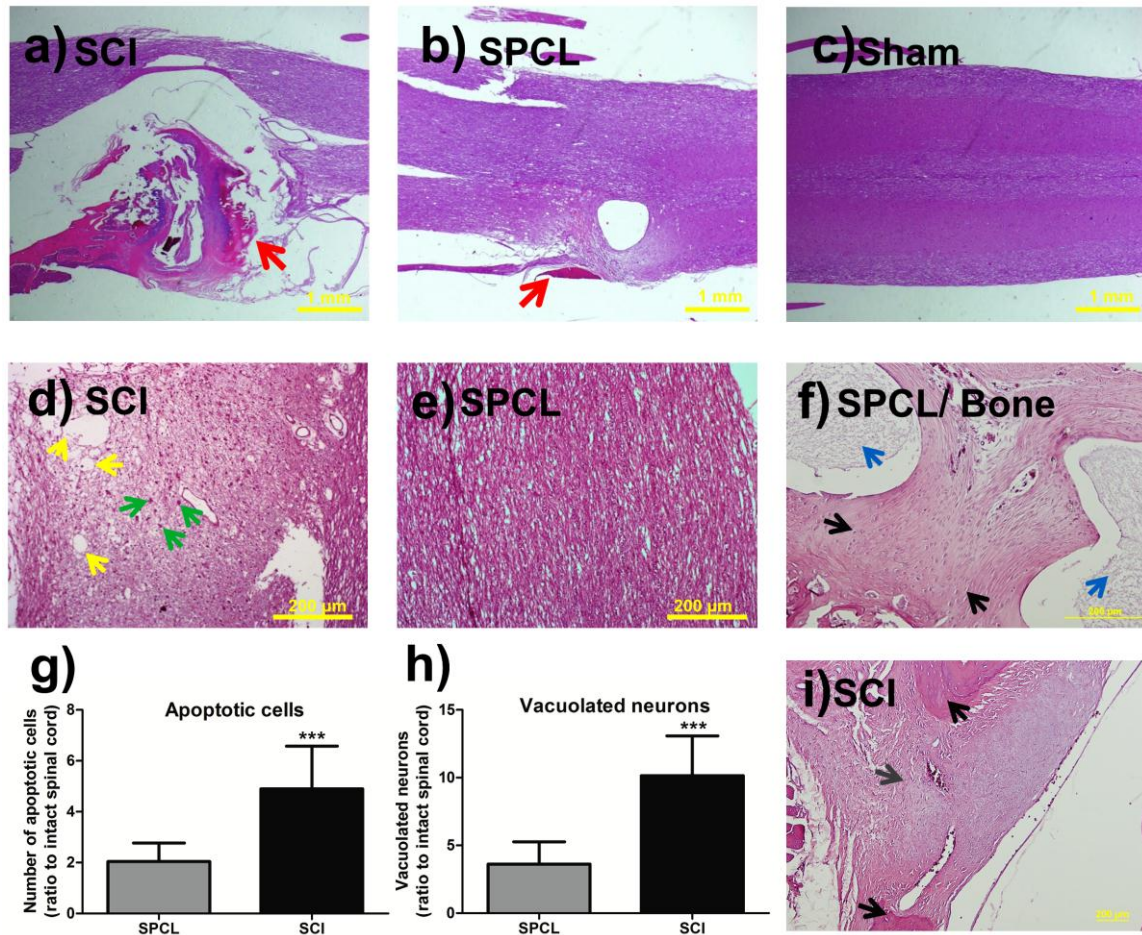


Figure IV.6. Histological analyses of spinal cord tissue and vertebra bone stained with Hematoxylin and Eosin. (a) Injured rats without spine stabilization (SCI) presented higher vertebral canal tissue infiltration (red arrows) than the ones with stabilization (SPCL) (b). The laminectomy alone did not affect the spinal cord of Sham animals (c). With higher magnification was possible to observe more cell death (green arrows pointing to chromatin condensation) and more vacuolated neurons (yellow arrows pointing to cyst-like structures) in rats without stabilization (d) than in those with with SPCL stabilization (e). In animals without stabilization both apoptotic cells (g) and vacuolated neurons (h) were present in greater numbers than in animals with stabilization. (e) At the vertebral level was possible to observe that newly bone was able to grow between the SPCL fibers (blue arrows). Moreover was possible to observe some signals of bone maturation, as the presence of lacunas, small spaces on bone matrix filled by osteocyte (black arrows). (i) In animals without SPCL implantation it was only possible to observe the formation of granulation tissue (gray arrow) and no bone formation. Scale bar of a), b) and c): 1 mm; scale bar of d), e), f) and i): 200 μ m; spinal cord figures are from dorsal region; values are shown as mean \pm SEM, *** $p < 0.001$.

4. Discussion

Biodegradable materials have been successfully used in various clinical applications. These materials were first introduced more than 30 years ago by Kulkarni and colleagues [18] for use as absorbable sutures. After that, absorbable polymers have been successfully employed for various methods of fixation for small bones fractures [19]. Recently these implants have been studied for spine surgery; nonetheless, the use of metallic spinal fixation is still the standard clinical practice [6]. Moreover, until now none of the developed biodegradable implants were specifically designed for spine stabilization after a SCI.

We show that spine stabilization is an important element in the therapeutic intervention of SCI whenever there is a laminectomy. In fact, animals subjected to spinal stabilization presented significant motor recovery. These animals were able to recover from no apparent movement on the left paw, after surgery, to extensive movement of all three joints in the hindlimb (7 on the BBB scale), at 12 weeks. On the other hand, animals without spinal stabilization by the SPCL scaffolds, only recovered from no apparent movement to slight/extensive movements of one joint (2 on the BBB scale). The BBB results here presented for injury animals without treatment are in concordance with other previously published experiments [20, 21]. However, others also report a greater motor recovery from control animals (between 6 and 8 on the BBB scale) [22, 23]. These variations may be explained by different protocols in the post-operative care performed by each lab. For instance, some authors use fibrin as a natural sealant or to stabilize tissue grafts within the spinal cord [24, 25]. That protocol may influence the motor recovery of control rats, given that, it was previously described that fibrin alone promotes tissue regeneration [26].

Due to the subjectivity inherent to BBB test, the motor function of the animals was also assessed by the rotarod and open field test methods. Analysis of motor coordination, balance, motor control and trunk stability were performed with these tests. Moreover, there is lower interference by the observer in rotarod and open field tests since the motor analysis is generated by software. The results from open field analyses showed that animals with spine stabilization were able to walk longer distances and execute a higher number of rearings than non-stabilized animals. Either distance or rearing performance is correlated with the motor improvements observed in the BBB test. Once again, the benefits of spine stabilization were confirmed. The greater amount of rearing activity may be correlated by an increase in trunk stability due to the implanted scaffolds. Moreover, the rearing behavior showed that the SPCL scaffolds are mechanically able to support the body weight of the animals when they stand up. Furthermore, we previously reported that rapid prototyping allow us to adjust the mechanical properties of SPCL scaffolds [10]. In this sense, the higher mechanical strength required in case of implantation in bigger animal models or in humans can be achieved. The mechanical properties of the SPCL scaffolds are also strongly linked to polymers degradation rate. Previous studies showed that SPCL present a slow degradation rate [27] and here we observed that they allow bone ingrowth; so, it is expectable that the vertebral bone is completely repaired after total reabsorption of the scaffold by the human body in order to avoid catastrophic failure. However, studies with longer implantation periods are still needed.

The results from rotarod revealed that motor coordination and balance are partially improved. To perform this test the rats had to walk over a constant moving rod in a coordinated way or they fall off the drum. The results showed that animals with the spine stabilized by the scaffolds, unlike the non-stabilized animals, were able to perform

a coordinated motor behavior at 4rpm. However, when the difficulty of the task increased to 8 and 12 rpm, all of the animals were not able to adequately perform the test. These results support the fact that stabilization provided by the implanted scaffold improves the motor function of SCI animals. Both the lack of coordination in more demanding tasks and the BBB score (just 7) corroborate the fact that spine stabilization should be combined with other strategies to promote nervous tissue repair.

The results presented here can be explained by the fact that the stabilization of the cord prevented further tissue damage derived from animal movements. Unlike humans, most SCI animal models perform body movements a few minutes after the surgery. However, these animals suffered a laminectomy which will provoke trunk instability and most of the times, trunk torsion. In this sense, prevention of vertebral column instability will avoid further mechanical impacts between bone and spinal cord. Moreover, SPCL scaffolds were implanted in the open wound, establishing a connection to the adjacent vertebral bone and decreasing the infiltration of connective tissue, as it was possible to observe by histological analyses. Thus, it is most likely that the conjugation of spine stabilization and reduction of connective tissue infiltration provided by SPCL scaffolds protects the spinal cord, leading to the decrease in cell death and a smaller amount of vacuolated neurons observed in the spinal cord tissue of the stabilized rats.

The locomotor behavior improvements herein described may be explained by spontaneous neural plasticity after SCI. Previous studies demonstrated central nervous tissue is able to undergo anatomical rearrangement after a SCI. For instance, Weidner and colleagues [28] verified that transection of the dorsally projecting corticospinal tract (CST) led to spontaneous sprouting of the ventral CST projection. Moreover, this plasticity was responsible for motor improvements in the forelimb. Additionally, Bareyre and co-workers [29] demonstrated that some of the transected CST axons that

would normally innervate lumbar segments sprouted into the cervical cord to innervate propriospinal neurons. This led to a novel, indirect motor pathway to lumbar motor circuits, which was responsible for motor recovery. In this sense, the protection against further damage to both damaged and intact fibers that the scaffold is able to provide after the SCI, seems to create a more suitable environment for plasticity to take place.

These results show a correlation between spine stabilization and motor recovery in SCI rats. However, after an overview of the literature, it was almost impossible to find references to spine stabilization after a SCI in rat/mouse animal models. With exception of the Tator lab, that use a metallic device [30], researchers rarely have tried to mimic the standard clinical practice after SCI. An interesting attempt to improve scaffold alignment after implantation in the spinal cord revealed that spine stabilization (using steel wires) prevents scoliosis and reduces kyphosis in SCI rats [31]. Unfortunately, the authors did not assess motor recovery by the BBB test; nonetheless, this study reinforces our conception that the absence of spine stabilization may jeopardize the regenerative process. In this sense, current and future attempts to develop a successful treatment for SCI [32] might have to take into account this process. It is important to refer that the choice of the biomaterial and 3D architecture of the stabilization device might be crucial, as the results herein reported suggest that a device which prevents surrounding connective tissue infiltration into the spinal cord and allows the vertebral bone to regenerate contributes to nervous tissue reorganization. Other tissue engineering approaches to promote spine regeneration are mainly focused on intervertebral disc regeneration [33]. However, Dong and coworkers [34] demonstrated that laminae of the vertebral arch can be successfully reconstructed using collagen scaffolds and bone marrow stromal cells. In a SCI situation, collagen scaffolds would have to be combined with metallic devices to promote spine stabilization, given that, collagen presents weak

mechanical properties. In any case, this is an interesting work showing that tissue engineering can successfully regenerate vertebral bone.

The hemisection model here employed preserve the integrity and function of one side of the cord which usually is sufficient to maintain bladder and bowel function resulting in less post-operative care and reduced animal death. Nonetheless, in future studies it will be important to analyze the benefits of spine stabilization in other models, namely the contusion model which reveal features similar to those seen in the clinical context. Moreover, future studies may also be focused on the use of mathematical models of human spine in order to study the applicability of SPCL scaffolds in patients. The finite element model (FEM) is the most common spine model and its incorporate realistic geometry of the vertebrae and physical properties of the soft tissue connecting the vertebrae [35]. With FEM it is possible to study kinematics (intervertebral motions), kinetics (motions in response to applied loads), and internal strains and stresses of the human spine [35]. In this sense, it would be possible to study the response of the SPCL scaffolds to mechanical stresses usually found in the human body. Additionally, the spine model may be combined with a SCI mathematical model, as the one described by Russell and colleagues [36], in order to further understand the neuroprotective effect of SPCL stabilization in a SCI situation.

In conclusion, in this work we present a biodegradable scaffold specifically designed for spine stabilization of SCI in the rat that can also provide support for bone ingrowth. More importantly, we also revealed that stabilization by SPCL scaffolds leads to an improvement of the motor skills of affected animals. Therefore, researchers currently testing treatments for SCI repair might have to take into account the use of spine stabilization in combination with their approaches. Further work will be focused on the

combination of spine stabilization and regeneration comprising SPCL scaffolds in combination with a hydrogel loaded with cells for SCI repair.

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References

- [1] Wyndaele M, Wyndaele JJ. Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? *Spinal Cord*. 2006;44:523-9.
- [2] Ouzký M. Towards concerted efforts for treating and curing spinal cord injury. *Concil of Europe*. 2002;Doc. 9401 27.
- [3] Dimar JR, Carreon LY, Riina J, Schwartz DG, Harris MB. Early Versus Late Stabilization of the Spine in the Polytrauma Patient. *Spine*. 2010;35:S187-S92.
- [4] Fehlings MG, Wilson JR. Timing of Surgical Intervention in Spinal Trauma What Does the Evidence Indicate? *Spine*. 2010;35:S159-S60.
- [5] Bracken MB. Methylprednisolone and Acute Spinal Cord Injury: An Update of the Randomized Evidence. *Spine*. 2001;26:S47-S54.
- [6] Vaccaro AR, Singh K, Haid R, Kitchel S, Wuisman P, Taylor W, et al. The use of bioabsorbable implants in the spine. *The Spine Journal*. 2003;3:227-37.
- [7] Cakir B, Richter M, Huch K, Puhl W, Schmidt R. Dynamic stabilization of the lumbar spine. *Orthopedics*. 2006;29:716-22.

- [8] Molinari RW. Dynamic stabilization of the lumbar spine. *Current Opinion in Orthopaedics*. 2007;18:215-20.
- [9] Goldstein IM, Agarwal N, Mammis A, Barrese J, Christiano LD. Rates of Infection with Dynamic Stabilization Compared to Posterior Instrumented Fusion. 28th Annual Meeting of the AANS/CNS Section on Disorders of the Spine and Peripheral Nerves. Orlando 2012.
- [10] Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Pedro AJ, Leite-Almeida H, et al. Development and Characterization of a Novel Hybrid Tissue Engineering Based Scaffold for Spinal Cord Injury Repair. *Tissue Engineering Part A*. 2010;16:45-54.
- [11] Rodrigues MT, Gomes ME, Viegas CA, Azevedo JT, Dias IR, Guzón FM, et al. Tissue-engineered constructs based on SPCL scaffolds cultured with goat marrow cells: functionality in femoral defects. *Journal of Tissue Engineering and Regenerative Medicine*. 2011;5:41-9.
- [12] Rada T, Santos TC, Marques AP, Correlo VM, Frias AM, Castro AG, et al. Osteogenic differentiation of two distinct subpopulations of human adipose-derived stem cells: an in vitro and in vivo study. *Journal of Tissue Engineering and Regenerative Medicine*. 2011.
- [13] Martins A, Chung S, Pedro AJ, Sousa RA, Marques AP, Reis RL, et al. Hierarchical starch-based fibrous scaffold for bone tissue engineering applications. *Journal of Tissue Engineering and Regenerative Medicine*. 2009;3:37-42.
- [14] Van Zutphen LFM, Baumans V, Beynen, AC. *Principles of Laboratory Animal Science*: Elsevier; 2001.
- [15] Basso DM, Beattie MS, Bresnahan JC. A Sensitive and Reliable Locomotor Rating-Scale for Open-Field Testing in Rats. *Journal of Neurotrauma*. 1995;12:1-21.
- [16] Sousa N, Almeida OFX, Wotjak CT. A hitchhiker's guide to behavioral analysis in laboratory rodents. *Genes, Brain and Behavior*. 2006;5:5-24.
- [17] Ross M, Kaye GI, Pawlina W. *Histology: A Text and Atlas*: Lippincott Williams & Wilkins; 2002.
- [18] Kulkarni RK, Pani KC, Neuman C, Leonard F. Polylactic Acid for Surgical Implants. *AMA Arch Surg*. 1966;93:839-43.
- [19] Bucholz RW, Henry S, Henley MB. Fixation With Bioabsorbable Screws For The Treatment Of Fractures Of The Ankle. *Journal of Bone and Joint Surgery-American Volume*. 1994;76A:319-24.

- [20] Hou T, Wu Y, Wang L, Liu Y, Zeng L, Li M, et al. Cellular Prostheses Fabricated with Motor Neurons Seeded in Self-Assembling Peptide Promotes Partial Functional Recovery After Spinal Cord Injury in Rats. *Tissue Engineering Part A*. 2011;974-85.
- [21] Zhang JF, Zhao FS, Wu G, Kong QF, Sun B, Cao J, et al. Therapeutic effect of co-transplantation of neuregulin 1-transfected-Schwann cells and bone marrow stromal cells on spinal cord hemisection syndrome. *Neuroscience Letters*. 2011;497:128-33.
- [22] Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, et al. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. *Proceedings of the National Academy of Sciences*. 2002;99:3024-9.
- [23] Li S, Strittmatter SM. Delayed Systemic Nogo-66 Receptor Antagonist Promotes Recovery from Spinal Cord Injury. *The Journal of Neuroscience*. 2003;23:4219-27.
- [24] Cheng H, Cao Y, Olson L. Spinal Cord Repair in Adult Paraplegic Rats: Partial Restoration of Hind Limb Function. *Science*. 1996;273:510-3.
- [25] Pritchard CD, Slotkin JR, Yu D, Dai H, Lawrence MS, Bronson RT, et al. Establishing a model spinal cord injury in the African green monkey for the preclinical evaluation of biodegradable polymer scaffolds seeded with human neural stem cells. *Journal of Neuroscience Methods*. 2010;188:258-69.
- [26] Sharp KG, Dickson AR, Marchenko SA, Yee KM, Emery PN, Laidm   I, et al. Salmon fibrin treatment of spinal cord injury promotes functional recovery and density of serotonergic innervation. *Experimental Neurology*. 2012.
- [27] Azevedo HS, Gama FM, Reis RL. In Vitro Assessment of the Enzymatic Degradation of Several Starch Based Biomaterials. *Biomacromolecules*. 2003;4:1703-12.
- [28] Weidner N, Ner A, Salimi N, Tuszynski MH. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. *Proceedings of the National Academy of Sciences*. 2001;98:3513-8.
- [29] Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. *Nat Neurosci*. 2004;7:269-77.
- [30] Nomura H, Katayama YM, Shoichet MS, Tator CH. Complete Spinal Cord Transection Treated by Implantation of A Reinforced Synthetic Hydrogel Channel Results in Syringomyelia and Caudal Migration of the Rostral Stump. *Neurosurgery*. 2006;59:183-92.

- [31] Rooney GE, Vaishya S, Ameenuddin S, Currier BL, Schiefer TK, Knight A, et al. Rigid Fixation of the Spinal Column Improves Scaffold Alignment and Prevents Scoliosis in the Transected Rat Spinal Cord. *Spine*. 2008;33:914-9.
- [32] Tohda C, Kuboyama T. Current and future therapeutic strategies for functional repair of spinal cord injury. *Pharmacology & Therapeutics*. 2011;132:57-71.
- [33] O'Halloran DM, Pandit AS. Tissue-engineering approach to regenerating the intervertebral disc. *Tissue Engineering*. 2007;13:1927-54.
- [34] Dong Y, Chen X, Wang M, Hong Y. Construction of Artificial Laminae of the Vertebral Arch Using Bone Marrow Mesenchymal Stem Cells Transplanted in Collagen Sponge. *Spine*. 2012;37:648-53.
- [35] Panjabi MM. Cervical Spine Models for Biomechanical Research. *Spine*. 1998;23:2684-99.
- [36] Russell CM, Choo AM, Tetzlaff W, Chung T-E, Oxland TR. Maximum Principal Strain Correlates with Spinal Cord Tissue Damage in Contusion and Dislocation Injuries in the Rat Cervical Spine. *Journal of Neurotrauma*. 2012;29:1574-85.

CHAPTER V

Interactions Between Schwann and Olfactory Ensheathing Cells with a
Starch/Polycaprolactone Scaffold aimed at Spinal Cord Injury Repair

Chapter V.

Interactions Between Schwann and Olfactory Ensheathing Cells with a Starch/Polycaprolactone Scaffold aimed at Spinal Cord Injury Repair*

Abstract

Spinal cord injury (SCI) represents a major world health problem. Therefore it is urgent to develop novel strategies that can specifically target it. We have previously shown that the implantation of starch-based scaffolds (SPCL) aimed for spine stabilization on SCI animals leads to motor skills improvements. Therefore, we hypothesize that the combination of these scaffolds with relevant cell populations for SCI repair will, most likely, lead to further improvements. Therefore, in this work, the ability of SPCL scaffolds to support the 3D culture of olfactory ensheathing cells (OECs) and Schwann cells (SCs) was studied and characterized. The results demonstrate for the first time that SPCL scaffolds were able to support the growth and migration of olfactory ensheathing cells (OECs) and Schwann cells. Moreover, the results indicate that two weeks of *in vitro* culture is the ideal time to reach a high number of transplantable cells. Future work will focus on the spine stabilization of SCI animals using SPCL scaffolds loaded with OECs or SCs for SCI regeneration.

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1. Introduction

Motivated by the physical consequences of the World War II, the era of cell transplantation began with the landmark work of Georges Mathé which transplanted bone marrow to six persons exposed to high doses of radiation [1]. More than sixty years later the hematopoietic stem cell transplantation still is the only one used in clinical practice. Nevertheless, cell transplantation approaches are, since then, being extensively investigated for a wide range of diseases/injuries. In the particular case of spinal cord injuries, a condition that affect millions all over the world [2], two kinds of transplanted cells gain ground when compared to the others. Olfactory Ensheathing Cells (OECs), obtained from olfactory bulbs or nasal mucosa, and Schwann Cells (SCs), found in the peripheral nervous system, have shown to promote neuronal repair, remyelination and functional recovery after transplantation into the spinal cord [3-7]. Both cell types are known to express similar molecular markers, such as p75 and S100 and to display a similar morphological phenotype in culture.[8, 9] However, contrasting with SCs, which are largely involved in myelination and repair of peripheral nerve fibers, the OECs are known to provide support and guidance to the growth of newly formed axons from the olfactory mucosa to the olfactory bulbs. Moreover, they are also known to be able migrate through the glial scar [10].

Recent modest results of clinical trials [11, 12] emphasize the need of further studies to understand the mechanism of action and transplantation of these cells. One of the problems associated with cell transplantation therapies is cell survival *in vivo*, since extensive cell death is usually observed after transplants [13, 14]. This fact is mainly related with the lack of matrix support of transplanted cells, as well as the inflammation in the local of injury [15]. For these reasons we propose the delivery of cells within a scaffold that is not implanted directly in the injury site, thus avoiding the cell death by

the lack of matrix support and decreasing the effects of inflammation on the fate of transplanted cells. Hence, within this work, we tested the ability of a previously developed and characterized natural starch-based scaffold (SPCL, starch poly- ϵ -caprolactone) [16] to support the 3D culture of OECs and SCs. We have previously shown significant motor recovery in SCI animals due the spine stabilization by using the above referred scaffolds [17]. Moreover, it was previously demonstrated that OECs and SCs are able to secrete neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor and glia cell line-derived neurotrophic factor [18, 19]. Additionally, the secretome of these two cells has previously shown to have a positive effect on axonal growth both *in vitro* [20-22] and *in vivo* [23-25]. Therefore the presence of these cells on the areas of the scaffold which face the spinal cord, will allow a localized deliver of neurotrophic factors to the injured area, fostering regeneration to occur. In this sense, combining the secretome effects of OECs and SCs with the previously observed beneficial effects of spine stabilization provided by the SPCL scaffolds implantation may lead to further locomotory recovery.

To do so, we first need to figure out if it is possible to culture OECs and SCs on the SPCL scaffolds. Therefore, in the present report we assessed the proliferation, metabolic activity, adhesion, morphology and distribution of primary OECs and SCs when cultured in a 3D fashion on SPCL scaffolds. The results revealed that SPCL scaffolds were able to support the growth and migration of both OECs and SCs. Moreover, we determined that two weeks of *in vitro* pre-incubation is the ideal time to achieve a higher number of cells on these scaffolds.

2. Materials & Methods

2.1. *Semi tubular SPCL scaffolds development*

The SPCL scaffolds were developed as previously reported by Silva *et al.* [16]. Briefly, porous sheets featuring inter filament orientations of 90° were produced by 3D plotting, a rapid prototyping technology (Bioplotter®, Envisiontec GmbH). Tubular scaffolds were obtained by rolling up porous sheets around a cylinder and subsequent heat treatment at 65° C during 30 min for inducing the adhesion between filaments. Two different porous tubular scaffold designs were obtained featuring a single or double layer, further referred as 90/1 and 90/2 for structures with one or two layers, respectively. After being processed and in order to acquire semi tubular form, the tubular scaffolds were cut in the middle with the aid of a blade.

2.2. *Scanning Electron Microscope Analyses*

Scanning electron microscopy (SEM) was used for analysis of morphology of the different SPCL tubular scaffolds. For this purpose, all samples were coated with Au/Pd via ion-sputtering prior to observation in a Stereoscan 360 scanning electron microscope (Leica Cambridge Co., Cambridge, United Kingdom).

2.3. *Cell Culture*

2.3.1. Isolation and culture of Olfactory Ensheathing Cells

OECs were harvested from olfactory bulbs of 4-days-old Wistar rats, according to the protocol described by Ramon-Cuétó *et al.* [26]. Briefly, upon dissection all meninges

were removed and the tissue was digested with 0,125% Collagenase (Sigma) for 20h at 37 °C. The digested tissue was mechanically dissociated with a pipette and then filtrated through a 70 µm cell strainer (BD Falcon). After centrifugation at 1000rpm for 10 min, cells were resuspended and plated in uncoated plates for 18h. A posterior change to uncoated plates for 36h was made, as it is expected that most of the fibroblasts and astrocytes will attach in the first and second period, respectively. Finally, cell suspensions were transferred to poly-D-lysine treaded flasks and cultured in DMEM/F12 (Gibco) with 10% of FBS (Gibco) and 1% of antibiotic-antimycotic solution (Sigma) at 37 °C and 5% CO₂ (v/v). OECs were then enriched by the supplementation with Bovine Pituitary Extract (5µg/ml, Gibco) and Forskolin (2µg/ml, Sigma). Cells were seeded into the scaffolds at passage 2, being that no trypsin was used and cells were detached using a cell scrapper.

2.3.2. Isolation and culture of Schwann Cells

SCs were obtained from sciatic nerves of 4-weeks-old male Wistar rats, according to the protocol described by Mauritz *et al.* [27]. Upon dissection, sciatic nerves were cut into 3 fragments each and incubated in DMEM-F-12 (Gibco no.11320-074), with 10% Fetal Bovine Serum (FBS, Gibco 10106-169), 1% Penicillin-Streptomycin antibiotic (Pen-Strep, Gibco no.15140-148), Forskolin (2µg/ml, Sigma) and Bovine Pituitary Extract (5µg/ml, Gibco). Following 7 days of incubation at 37°C and 5% CO₂, the nerve fragments were enzymatically dissociated with Collagenase solution (320U/ml) (Sigma no.C0130-1G) containing 5U/ml of DNase (Worthington Lot 51M5139) for 20 hours. After mechanical dissociation, the collagenase was removed by repeated

centrifugations. Cells were then allowed to grow in Poly-D-Lysine coated flasks. As for OECs, SCs were also seeded into the scaffolds at passage 2.

2.4. Immunocytochemistry

Immunostaining was performed as follows. SC or OEC-covered cover-slips were fixed in 4% paraformaldehyde for 20 min and then washed with PBS. After cell blocking by treating them with 10% of FCS in PBS (Gibco) for 1 h, Rabbit anti-S100 primary antibody (Invitrogen) was incubated for 1h. After washing with PBS cells were exposed to the secondary antibody Alexa Fluor 488 rabbit anti-rat IgG (Molecular Probes) for 1 h. Finally, cell nuclei were counterstained with DAPI (1µg/ml, ThermoScientific) for 5 min and washed with PBS. Primary antibody was omitted to produce negative controls. Samples were observed under an Olympus BX-61 Fluorescence Microscope (Olympus).

2.5. Seeding of OECs or SCs on SPCL scaffolds

To ensure adherence of cells, SPCL scaffolds were coated with poly-L-lysine before cell seeding. Cells were centrifuged at 1200 rpm for 5 min and then resuspended in a small volume of medium to yield a solution with a density of 5×10^4 cell/10µl. Subsequently, scaffolds were transferred to empty wells and 10µl of cell suspension were dropped on the top of the inner lumen of the semi tubular scaffolds. After an incubation of 2 h in a humidified atmosphere at 37 °C and 5% CO₂, SPCL scaffolds were transferred to new wells containing complete medium (for composition details refer to OECs or SCs cell culture section) and incubated for 2, 7, 14 and 21 days for the *in vitro* assays.

2.6. *Proliferation Evaluation*

In the time points mentioned above, proliferation, through double-stranded DNA quantification, was assessed. The fluorescent dye Picogreen was used to acquire the total amount of dsDNA [28]. Briefly, after cell lysis by osmotic and thermal shock three components of QuantiTTM PicoGreen dsDNA Assay Kit (Invitrogen), Tris-HCl-EDTA (50%), Picogreen dye (35,45%) and cell lysated (16,35%), were mixed in an opaque 96 well plate. The fluorescent intensity, proportional to the amount of dsDNA, was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm, in a microplate reader (Bio-Tek). The dsDNA concentration for triplicates of each sample (n=3) was calculated using a standard curve relating quantity of dsDNA and fluorescence intensity.

2.7. *Metabolic Activity Assay*

Cell viability was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). This assay is based on the bioreduction of a tetrazolium compound (MTS), into a water-soluble brown formazan product. This conversion is accomplished by NADPH or NADH production by the dehydrogenase enzymes in metabolically active cells. Complete culture medium was replaced by standard DMEM/F12 medium containing MTS in a 5:1 ratio and incubated in a humidified atmosphere at 37 °C and 5% CO₂. After 3h of incubation, the optic density for triplicates of each sample (n=3) was measured at 490 nm in a microplate reader.

2.8. *Assessment of cell adhesion, morphology and distribution*

At each defined time culture period, the SPCL scaffolds were fixed with 4% of paraformaldehyde for 30 min at room temperature. Then, 0.1µg/ml of Phalloidin (Sigma) was added to the cells during 30 min. After washing 3 times with PBS, cell nuclei were counterstained with DAPI (1µg/ml, ThermoScientific) for 5 min. The SPCL scaffolds were then observed by confocal microscopy (Olympus).

2.9. *Statistical analysis*

To assess the statistical differences on metabolic activity and proliferation of OECs and SCs it was performed a 'two way ANOVA' test followed by a Bonferroni post-test. Statistical significance was defined for $p < 0.05$.

3. Results and discussion

Ensure cell survival *in vivo* is one of the problems associated with cell transplantation therapies, since extensive cell death is usually observed after transplants [13]. The lack of matrix support, ischemia and local inflammation are some of the factors related with poor cell survival after cell based therapies [15]. Typical cells preparations for injection imply enzymatic digestion of the matrix. When cells that normally grow in attachment are kept in suspension, a pathway of cell death called anoikis is initiated [29]. Anoikis, a greek word for homelessness, is described in cell biology as programmed cell death generated by loss of matrix attachments [30]. In addition, it is well known that after SCI ischemia and inflammation occur [31]. These two events generate oxygen free radicals and inflammatory cytokines that can directly damage graft cells via cell membrane damage or trigger signaling pathways that result in caspase activation and cell death

[15]. In order to cell-based regenerative medicine approaches become a successful therapy, these issues should be addressed. Okano *et al.* [32, 33], proposed the use of cell sheets, avoiding in this way the loss of cell matrix support. Others targeted specific molecular pathways aimed at cell death blockage [34]. We consider that the delivery of cells, with a trophic ability, within a scaffold that is not implanted directly in the injury will avoid the cell death by anoikis and decrease the effects of inflammation on the fate of transplanted cells.

We have recently shown that the spine stabilization by SPCL scaffolds implanted in the vertebral column of SCI rats lead to significant improvements [17]. However, the spine stabilization alone will not successfully treat the injured animals, and therefore further actions are needed to repair the spinal cord. Thus, in this work we investigated the ability of SPCL scaffolds to support cells aimed for SCI repair. In this way the scaffolds will act as a stabilization device and at same time will allow a localized deliver of neurotrophic factors to the injured spinal cord produce by the OECs or SCs.

3.1. 3D culture of OECs on SCPL scaffolds

OECs and SCs were analyzed, prior seeding on SPCL scaffolds, by immunocytochemistry against S100 receptor (Figure 1a and 1b). Subsequently, cells were seeded in the inner core of SPCL scaffolds with one layer (90/1, Figure 1c) or two layers (90/2, Figure 1d). Cell adhesion, migration, proliferation and metabolic activity were assessed 2, 7, 14 and 21 days after the seeding.

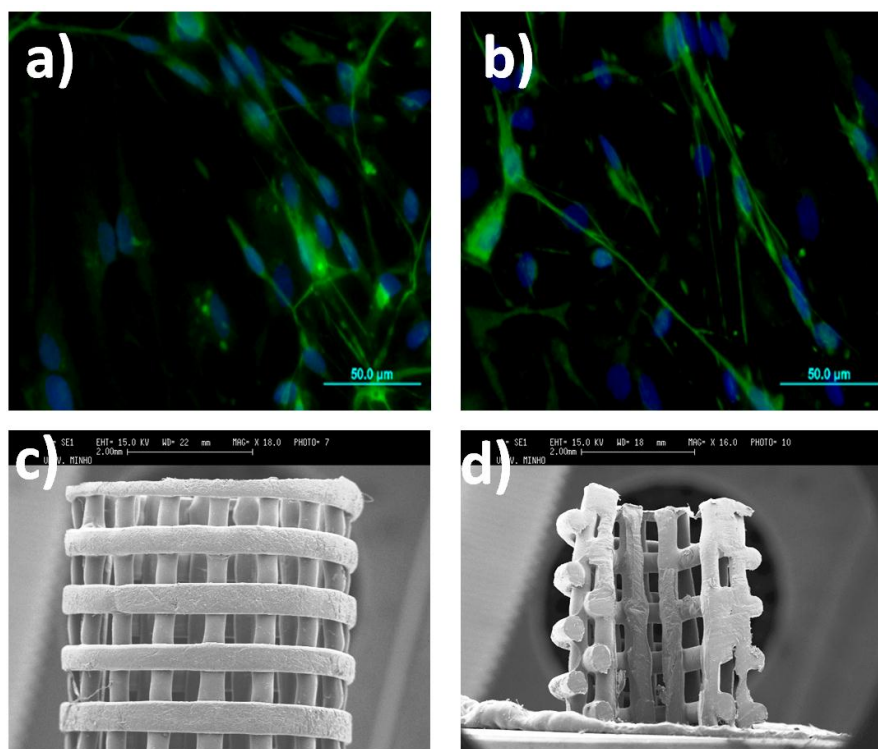


Figure V.1. Cells and scaffolds before the 3D cultured. The presence of OECs (a) and SCs (b) after cell isolation was confirmed by immunocytochemistry against S-100 protein (green) and nuclei counterstained with DAPI (blue). OECs and SCs were then seeded on the inner core of single (c) or double (d) layer SPCL scaffolds.

The quantification of dsDNA revealed a significant increase of cell proliferation from day 2 to day 21, both on single and double layer SPCL scaffolds (Figure 2a). Furthermore, only at the second day of culture, the quantity of OECs on single layer scaffolds is significantly higher than in double layer scaffolds, demonstrating a more efficient cell seeding on 90/1 scaffolds. Interesting, either for 90/1 and 90/2 scaffolds, the quantity of dsDNA did not increase from day 2 to 1 week in culture, which indicates that these cells need a time to adapt to the new 3D surface. Moreover, cells cultured on single layer scaffolds did not presented significant cell proliferation between 2 and 3 weeks. On the contrary on 90/2 scaffolds dsDNA significant increases between 2 and 3 weeks were observed. These differences can be explained by the lower porosity of the

90/2 scaffolds. We have previously reported differences in the porosity of single and double layer scaffolds, being 90/2 scaffolds 15% less porous than the 90/1 ones [16]. So that in this condition the cells need more time to reach the confluence. The dsDNA quantification results are in concordance with the measured metabolic activity of OECs (Figure 2b), which from day 2 to day 21 increased significantly on both types of scaffolds. Moreover and up to 14 days in culture, OECs seeded on 90/1 scaffolds showed a statistically higher metabolic activity than the ones seeded in 90/2 scaffolds. As referred before the cell seeding was more efficient on these scaffolds than on the 90/2 ones, a fact that is closely related with these observations, as the initial cell densities would be necessarily different in both. Then, again, the metabolic activity is significantly different between 2 and 3 weeks for double layer scaffolds but not for single layer.

The assessment of migration, adhesion and morphology of OECs by confocal microscopy showed a low efficiency on cell seeding (Figure 2c and 2d, 48h). However, over time the OECs successfully colonized the entire SPCL fibers, showing that these cells are capable to migrate and grow in a 3D manner on SPCL scaffolds (Figure 2c and 2d). Moreover, after 2 weeks it was possible to observe the typical spindle-shaped morphology of OECs, which confirmed how well adapted the cells were. Finally, it is possible to confirm that in 90/1 scaffolds cells were confluent at 2 weeks, but for 90/2 the confluence was only reached at 3 weeks. All of the tests indicated that OECs culture on SPCL scaffolds with one or two layers should be implanted in animals after 2 or 3 weeks of *in vitro* culture.

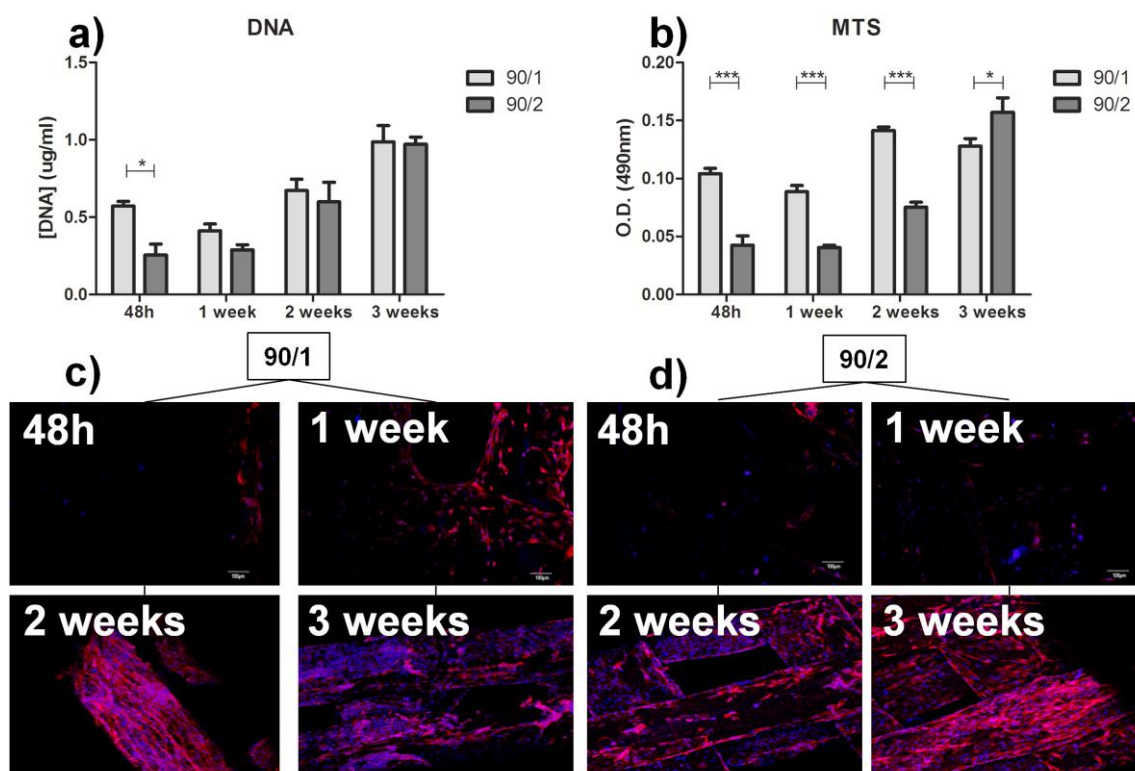


Figure V.2. 3D cell culture of OECs on SPCL scaffolds. A significant ($p < 0.05$) overall increase over time was observed either for (a) dsDNA quantification or (b) MTS test. Cellular adhesion, distribution and morphology were assessed by cytoplasmic staining with phalloidin (red) and nuclei counterstained with DAPI (blue), followed by confocal observation. Despite of a low efficient cell seeding (48h), OECs were able to migrate and colonize all fibers of both single (c) and double (d) layer SPCL scaffold. Values are shown as mean \pm SEM ($n=3$).

3.2. 3D culture of SCs on SCPL scaffolds

Contrasting with OECs, SCs adapted faster on the rigid 90/2 scaffold than in the more porous 90/1 scaffold. dsDNA quantification test revealed that as OECs, the SCs also significantly increased the number of cells from day 2 to day 21 in both types of scaffolds (Figure 3a). Again, as happened with OECs, the SCs also took around one week to adapt to the new surface, since no significant differences were detected between 2 days and 1 week of culture. However, and in contrast to OECs, the quantity of dsDNA on SCs culture is always higher in the double layer scaffolds, showing that these cells

were better adapted to 90/2 scaffolds. Interestingly, cell proliferation stops at 2 weeks for both types of scaffolds, indicating that the implantation of SPCL scaffolds with SCs should be done after around 2 weeks of *in vitro* culture. By analyzing the metabolic activity of SCs cultured on SPCL scaffolds (Figure 3b) it was possible to confirm that the cells took around one week to be adapted to the scaffold (no differences between 2 days and 1 week). No differences were found between weeks 2 and 3, reinforcing the idea that these cells should only be cultured on the scaffolds for periods up to two weeks before transplantation. It is surprising, though, the absence of significant differences between the one and two layers scaffolds; in other words it seems that despite the lower number of cells in the one layer scaffolds, they were more metabolically active than the cells from two layers, a difference that may be due to the higher porosity of the one layer scaffolds.

The assessment of migration, adhesion and morphology of SCs by confocal microscopy showed a low efficient cell seeding, particular for single layer scaffolds (Figure 3c, 48h). However, over time the SCs, as happened with OECs, also successfully colonized the entire SPCL fibers (Figure 3c and 3d), showing that these cells are capable to migrate and grow in a 3D manner on SPCL scaffolds. Finally, it was possible to confirm that cells reached the confluence at 2 weeks either in 90/1 and 90/2 scaffolds, and there was a decrease in cell number at 3 weeks. All of the tests indicated that SCs culture on SPCL scaffolds with one or two layers should be implanted in animals after 2 weeks of *in vitro* culture.

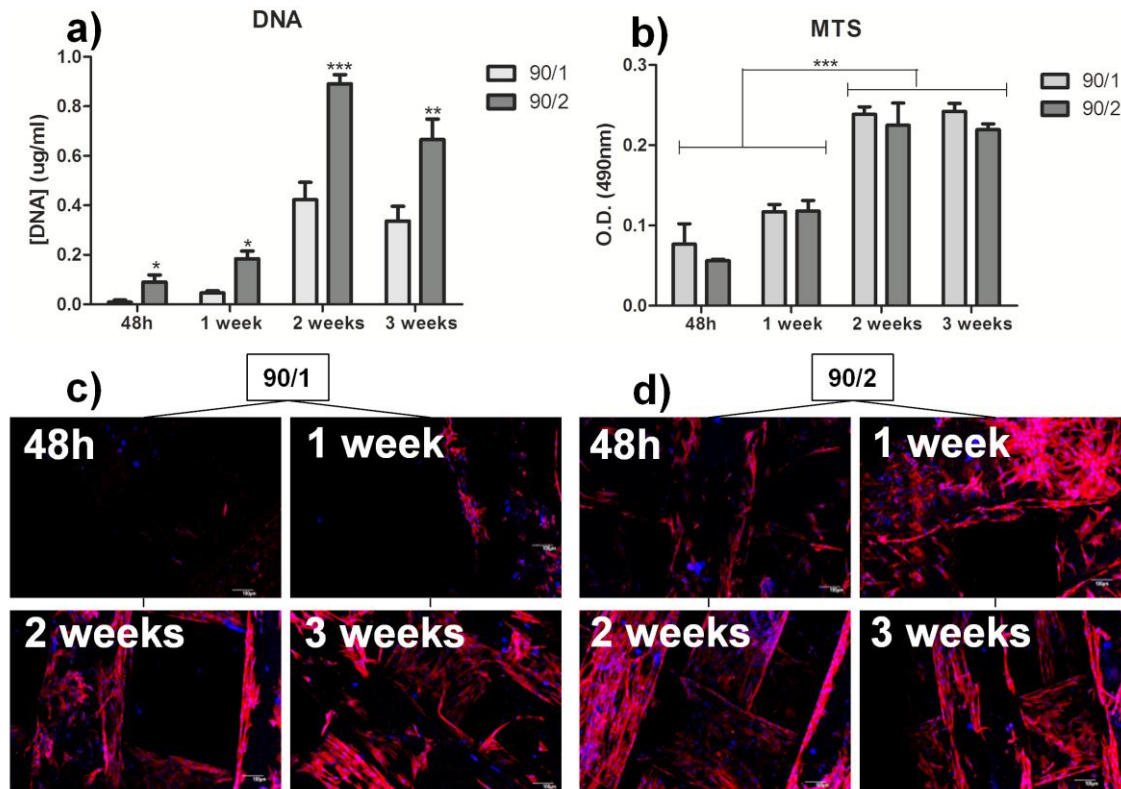


Figure V.3. 3D cell culture of SCs on SPCL scaffolds. Both (a) cell proliferation and (b) metabolic activity analyses revealed a significant ($p < 0.05$) overall increase over time. Confocal observation after cytoplasmic staining with phalloidin (red) and nuclei counterstained with DAPI (blue), revealed low efficient cell seeding (48h) particular for single layer scaffolds(c) than for double layer (d). Over time, as happened with OECs, SCs were also able to migrate and colonize all fibers of both SPCL scaffolds. Values are shown as mean \pm SEM ($n=3$).

The results presented in this work show that OECs and SCs can successfully grow and fully colonize both single and double SPCL scaffolds. In fact, in both cases there are some inconsistencies between the dsDNA assay and the metabolic activity of the cells. However, it is important to notice that as previously described by Hutmacher *et al.* [35] the quantification of metabolic activity in three dimensions cultures by MTS test cannot be directly compared with proliferations assays, such as the dsDNA assays. Actually, while the latter are based on the Picogreen assay, which is a sensitive test that can be correlated with proliferation of cells with time, MTS relies only in the capability of cells

to metabolize MTS into a formazan salt, measuring the metabolic viability. This metabolic viability cannot be directly compared to cell proliferation as the cells own basal metabolism may be altered due to different parameters (such as: porosity, oxygen and nutrients availability, nature of the substrate where they are growing, etc.) that may or, may not, affect cell proliferation. Thus it is possible to obtain different trends for both assays, which do not necessarily invalidate the results obtained from them, as they do measure different parameters when cells are growing in 3D scaffolds. In this sense, the results presented in our work show, for OECs, that after 3 weeks of culturing the use of 90/1 or 90/2 scaffolds will not significantly affect the number of the transplanted cells. However, for SCs the amount of dsDNA is always significantly higher in the 90/2 scaffolds than in 90/1 ones. This means that for these cells it would be better to use the 90/2 scaffolds for implantation. Concerning the metabolic activity, the results show some differences in the initial time points analyzed, and the cells reach the maximum activity after 2/3 weeks of culture. Only in the last time points it is possible to obtain similar results in the metabolic activity assay between single and double layers scaffolds. However, it is important to notice that the key time points for transplantation presented here can only be used for these scaffolds, since, most likely cells will respond in different ways to different polymers. Nonetheless, as happened with the starch-based scaffolds presented in this work, several others studies had shown either for OECs or SCs that these cells also need an initial time to adapt to scaffolds made from different origins [36-39]. However, other studies, in which scaffolds were seeded with cells and implanted in animals just after a short pre-incubation *in vitro* of 1-2 days suggest the opposite [40-42]. Nevertheless, it seems reasonable that the study of how cells behave on scaffolds should be performed at longer time points before the *in vivo* implantation, since the implantation of a scaffold with a higher number of cells will most likely result

in a higher number of cell survival and in this way, increase the cells' related effects on the implanted tissue.

4. Conclusions

Within the present work it was possible to demonstrate for the first time that a starch-based scaffold was able to support the growth and migration of OECs and SCs. Moreover we demonstrated the importance of studying the long time *in vitro* incubation periods on the number of cells that could be transplanted within the scaffold. For the structures here in studied, we demonstrated that two weeks of culture is the ideal time to reach a high number of transplantable cells. Further work will focus on the spine stabilization of SCI animals using SPCL scaffolds loaded with OECs, SCs or a combination of both.

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References

- [1] Mathé G, Jammet H, Pendie N. Transfusions et greffes de moelle osseuse homologue chez des humaine irradiées a haute dose accidentellement. *Nouvelle rev franc hematol.* 1959;4.
- [2] Wyndaele M, Wyndaele JJ. Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? *Spinal Cord.* 2006;44:523-9.
- [3] Lu J, Féron F, Mackay-Sim A, Waite PME. Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. *Brain.* 2002;125:14-21.
- [4] Santos-Benito FF, Ramón-Cueto A. Olfactory ensheathing glia transplantation: A therapy to promote repair in the mammalian central nervous system. *The Anatomical Record Part B: The New Anatomist.* 2003;271B:77-85.
- [5] Fouad K, Schnell L, Bunge MB, Schwab ME, Liebscher T, Pearse DD. Combining Schwann Cell Bridges and Olfactory-Ensheathing Glia Grafts with Chondroitinase Promotes Locomotor Recovery after Complete Transection of the Spinal Cord. *The Journal of Neuroscience.* 2005;25:1169-78.
- [6] Andrews MR, Stelzner DJ. Evaluation of Olfactory Ensheathing and Schwann Cells after Implantation into a Dorsal Injury of Adult Rat Spinal Cord. *Journal of Neurotrauma.* 2007;24:1773-92.
- [7] Lavdas AA, Chen J, Papastefanaki F, Chen S, Schachner M, Matsas R, et al. Schwann cells engineered to express the cell adhesion molecule L1 accelerate myelination and motor recovery after spinal cord injury. *Experimental Neurology.* 2010;221:206-16.
- [8] Xu X, Onifer SM. Transplantation-mediated strategies to promote axonal regeneration following spinal cord injury. *Respiratory Physiology & Neurobiology.* 2009;169:171-82.
- [9] Wewetzer K, Verdú E, Angelov D, Navarro X. Olfactory ensheathing glia and Schwann cells: two of a kind? *Cell and Tissue Research.* 2002;309:337-45.
- [10] Zhang N, Yan H, Wen X. Tissue-engineering approaches for axonal guidance. *Brain Research Reviews.* 2005;49:48-64.
- [11] Huang HY, Chen L, Wang HM, Xiu B, Li BC, Wang R, et al. Influence of patients' age on functional recovery after transplantation of olfactory ensheathing cells into injured spinal cord injury. *Chinese Medical Journal.* 2003;116:1488-91.

- [12] Dobkin BH, Curt A, Guest J. Cellular Transplants in China: Observational Study from the Largest Human Experiment in Chronic Spinal Cord Injury. *Neurorehabilitation and Neural Repair*. 2006;20:5-13.
- [13] Yasuda T, Weisel RD, Kiani C, Mickle DAG, Maganti M, Li R. Quantitative analysis of survival of transplanted smooth muscle cells with real-time polymerase chain reaction. *The Journal of Thoracic and Cardiovascular Surgery*. 2005;129:904-11.
- [14] Hayashi M, Li T, Ito H, Mikamo A, Hamano K. Comparison of Intramyocardial and Intravenous Routes of Delivering Bone Marrow Cells for the Treatment of Ischemic Heart Disease: An Experimental Study. *Cell Transplantation*. 2004;13:639-47.
- [15] Robey TE, Saiget MK, Reinecke H, Murry CE. Systems approaches to preventing transplanted cell death in cardiac repair. *Journal of Molecular and Cellular Cardiology*. 2008;45:567-81.
- [16] Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Pedro AJ, Leite-Almeida H, et al. Development and Characterization of a Novel Hybrid Tissue Engineering Based Scaffold for Spinal Cord Injury Repair. *Tissue Engineering Part A*. 2010;16:45-54.
- [17] Silva NA, Sousa RA, Oliveira JT, Fraga JS, Fontes M, Cerqueira R, et al. Motor Improvements after Spine Stabilization of Spinal Cord Injured Rats with a Novel 3D Natural based Biodegradable Scaffold. *LisboaMay*, 2011.
- [18] Woodhall E, West AK, Chuah MI. Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors. *Molecular Brain Research*. 2001;88:203-13.
- [19] Assouline JG, Bosch P, Lim R, Kim IS, Jensen R, Pantazis NJ. Rat astrocytes and Schwann cells in culture synthesize nerve growth factor-like neurite-promoting factors. *Brain Research*. 1987;428:103-18.
- [20] Cao L, Mu L, Qiu Y, Su Z, Zhu Y, Gao L, et al. Diffusible, membrane-bound, and extracellular matrix factors from olfactory ensheathing cells have different effects on the self-renewing and differentiating properties of neural stem cells. *Brain Research*. 2010;1359:56-66.
- [21] Jiao Y, Novozhilova E, Karlén A, Muhr J, Olivius P. Olfactory ensheathing cells promote neurite outgrowth from co-cultured brain stem slice. *Experimental Neurology*. 2011;229:65-71.
- [22] Taylor JSH, Bampton ETW. Factors secreted by Schwann cells stimulate the regeneration of neonatal retinal ganglion cells. *Journal of Anatomy*. 2004;204:25-31.

- [23] Li Y, Field PM, Raisman G. Regeneration of Adult Rat Corticospinal Axons Induced by Transplanted Olfactory Ensheathing Cells. *The Journal of Neuroscience*. 1998;18:10514-24.
- [24] Li Y, Field PM, Raisman G. Repair of Adult Rat Corticospinal Tract by Transplants of Olfactory Ensheathing Cells. *Science*. 1997;277:2000-2.
- [25] Papastefanaki F, Chen J, Lavdas AA, Thomaidou D, Schachner M, Matsas R. Grafts of Schwann cells engineered to express PSA-NCAM promote functional recovery after spinal cord injury. *Brain*. 2007;130:2159-74.
- [26] Ramón-Cueto A, Nieto-Sampedro M. Glial cells from adult rat olfactory bulb: Immunocytochemical properties of pure cultures of ensheathing cells. *Neuroscience*. 1992;47:213-20.
- [27] Mauritz C, Grothe C, Haastert K. Comparative study of cell culture and purification methods to obtain highly enriched cultures of proliferating adult rat Schwann cells. *Journal of Neuroscience Research*. 2004;77:453-61.
- [28] Schofield GG. PicoGmeter, a custom-made fluorometer for the quantification of dsDNA by PicoGreen (R) fluorescence. *Biotechniques*. 2004;37:778-82.
- [29] Zvibel I, Smets F, Soriano H. Anoikis: Roadblock to Cell Transplantation? *Cell Transplantation*. 2002;11:621-30.
- [30] Reddig P, Juliano R. Clinging to life: cell to matrix adhesion and cell survival. *Cancer and Metastasis Reviews*. 2005;24:425-39.
- [31] Hulsebosch CE. Recent Advances in Pathophysiology and Treatment of Spinal Cord Injury. *Advances in Physiology Education*. 2002;26:238-55.
- [32] Yang J, Yamato M, Kohno C, Nishimoto A, Sekine H, Fukai F, et al. Cell sheet engineering: Recreating tissues without biodegradable scaffolds. *Biomaterials*. 2005;26:6415-22.
- [33] Pirraco RrP, Obokata H, Iwata T, Marques AP, Tsuneda S, Yamato M, et al. Development of Osteogenic Cell Sheets for Bone Tissue Engineering Applications. *Tissue Engineering Part A*. 2011:Epub ahead of print.
- [34] Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte Grafting for Cardiac Repair: Graft Cell Death and Anti-Death Strategies. *Journal of Molecular and Cellular Cardiology*. 2001;33:907-21.
- [35] Ng KW, Leong DTW, Hutmacher DW. The Challenge to Measure Cell Proliferation in Two and Three Dimensions. *Tissue Engineering*. 2005;11:182-91.

- [36] Mollers S, Heschel I, Damink LH, Schugner F, Deumens R, Moller B, et al. Cytocompatibility of a Novel, Longitudinally Microstructured Collagen Scaffold Intended for Nerve Tissue Repair. *Tissue Engineering Part A*. 2009;15:461-72.
- [37] Shen Y, Qian Y, Zhang H, Zuo B, Lu Z, Fan Z, et al. Guidance of Olfactory Ensheathing Cell Growth and Migration on Electrospun Silk Fibroin Scaffolds. *Cell Transplantation*. 2010;19:147-57.
- [38] Novikova LN, Pettersson J, Brohlin M, Wiberg M, Novikov LN. Biodegradable poly-[beta]-hydroxybutyrate scaffold seeded with Schwann cells to promote spinal cord repair. *Biomaterials*. 2008;29:1198-206.
- [39] Gupta D, Venugopal J, Prabhakaran MP, Dev VRG, Low S, Choon AT, et al. Aligned and random nanofibrous substrate for the in vitro culture of Schwann cells for neural tissue engineering. *Acta Biomaterialia*. 2009;5:2560-9.
- [40] Oudega M, Gautier SE, Chapon P, Fragoso M, Bates ML, Parel J-M, et al. Axonal regeneration into Schwann cell grafts within resorbable poly([alpha]-hydroxyacid) guidance channels in the adult rat spinal cord. *Biomaterials*. 2001;22:1125-36.
- [41] Hurtado A, Moon LDF, Maquet V, Blits B, Jérôme R, Oudega M. Poly (d,l-lactic acid) macroporous guidance scaffolds seeded with Schwann cells genetically modified to secrete a bi-functional neurotrophin implanted in the completely transected adult rat thoracic spinal cord. *Biomaterials*. 2006;27:430-42.
- [42] Moore MJ, Friedman JA, Lewellyn EB, Mantila SM, Krych AJ, Ameenuddin S, et al. Multiple-channel scaffolds to promote spinal cord axon regeneration. *Biomaterials*. 2006;27:419-29.

CHAPTER VI

The Effects of Peptide Modified Gellan Gum and Olfactory Ensheathing

Glial Cells on Neural Stem/Progenitor Cell Fate

Chapter VI.

The Effects of Peptide Modified Gellan Gum and Olfactory Ensheathing Glial Cells on Neural Stem/Progenitor Cell Fate.*

Abstract

The regenerative capacity of injured adult central nervous system (CNS) tissue is very limited. Specifically, traumatic spinal cord injury (SCI) leads to permanent loss of motor and sensory functions below the site of injury, as well as other detrimental complications. A potential regenerative strategy is stem cell transplantation; however, cell survival is typically less than 1%. To improve cell survival, stem cells can be delivered in a biomaterial matrix that provides an environment conducive to survival after transplantation. One major challenge in this approach is to define the biomaterial and cell strategies in vitro. To this end, we investigated both peptide-modification of gellan gum and olfactory ensheathing cells (OECs) on neural stem/progenitor cell (NSPC) fate. To enhance cell adhesion, the gellan gum (GG) was modified using Diels-Alder click chemistry with a fibronectin-derived synthetic peptide (GRGDS). Amino acid analysis demonstrated that approximately 300 nmol of GRGDS was immobilized to each mg of GG. The GG-GRGDS had a profound effect on NSPC morphology and proliferation, distinct from that of NSPCs in GG alone, demonstrating the importance of GRGDS for cell-GG interaction. To further enhance NSPC survival and outgrowth, they were cultured with OECs. Here NSPCs interacted extensively with OECs, demonstrating significantly greater survival and proliferation relative to monocultures

of NSPCs. These results suggest that this co-culture strategy of NSPCs with OECs may have therapeutic benefit for SCI repair.

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1. Introduction

Traumatic spinal cord injury (SCI) usually leads to significant neurological deficits and disabilities that result in loss of sensory and motor function, termed paraplegia or tetraplegia depending on the site of injury. This can subsequently result in other related problems such as infections of the bladder and kidneys and dysfunction of the bowel, as well as heart and respiratory system. All of these problems have a negative effect on the physiological, psychological and social behavior of SCI patients. Current clinical approaches are limited and mainly based on the use of anti-inflammatory agents, such as methylprednisolone [1]; however, its use is controversial as recent studies failed to reveal conclusive beneficial outcomes [2]. Some of the other strategies that have been tested clinically include: minocycline [3], anti-NogoA [4] and transplantation of oligodendrocyte precursor cells [5]. The latter trial was recently cancelled, underlying the urgent need to develop therapeutic strategies that can promote regeneration after SCI.

One approach currently under investigation for regeneration following SCI is the transplantation of cells into the spinal cord. Several groups have reported that the injection of cells, such as olfactory ensheathing cells (OECs) [6, 7], neural stem/progenitor cells (NSPCs) [8] or mesenchymal stem cells [9, 10], leads to motor improvements and/or tissue repair in SCI animal models. OECs are compelling because they support and guide olfactory axons [11], are able to grow through the glial scar [12] and secrete several neurotrophic factors [13]. NSPCs transplants have shown some functional repair, taking advantage of their ability to differentiate to neurons, oligodendrocytes and astrocytes [14]. However, to date no single repair strategy has successfully induced full functional recovery following SCI. Extensive cell death of the

transplanted cells after injection, due to inflammation and/or the absence of matrix support [15, 16], has limited the therapeutic benefit. To enhance cell survival, we investigated the co-delivery of two cell types in an engineered matrix where the second cell type provides trophic support and the matrix contributes to overcoming cell death through anoikis [17].

Biomaterials have been designed as vehicles for cell transplantation in order to enhance cell survival after transplantation. Hydrogels are particularly appealing for soft tissue applications because they can be designed to match the mechanical properties and water content of these tissues. For example, delivering NSPCs in chitosan tubular scaffolds demonstrated NSPC survival and promising results for tissue and functional repair [8]. The gellan gum hydrogel is compelling because it can be injected in a minimally-invasive way to form a gel in situ. Gellan gum (GG) is a linear anionic microbial polysaccharide composed of repeating units of glucose, glucuronic acid and rhamnose [18] and thus has multiple hydroxyl groups available for chemical modification. It has been studied for drug delivery and cartilage regeneration [19-21] and is approved by the FDA as a food additive.

In order to better mimic the extracellular matrix (ECM), biomaterials have been modified with several peptide sequences [22-24] to influence biological processes, such as cell adhesion, growth and development [25, 26]. Cells transplanted in a material that mimics both the mechanical and chemical properties of native tissue have been shown to be more efficacious after transplantation [27, 28].

Here we aim to synthesize GG with enhanced cell-ECM and cell-cell interactions for ultimate use in cell transplantation. To promote cell survival and interaction with gellan gum, we chemically conjugated the fibronectin-derived peptide sequence GRGDS via

Diels-Alder click chemistry and studied the impact of GRGDS-modified gellan gum (GG-GRGDS) on cell fate. To enhance cell survival beyond that provided by the ECM-mimetic peptide sequence, cell-cell interactions are investigated. Since OECs have been shown to provide guidance pathways to axons and NSPCs have been shown to promote some functional recovery, we investigated the co-culture of these two cell types to determine whether the OEC affected NSPC survival and/or differentiation. To gain a greater appreciation of the OEC-NSPC interaction, the two cell types were cultured in direct contact, in a transwell system or in the GG-GRGDS. Importantly, both OECs and NSPCs can be isolated from human patients [29, 30], which enhances the eventual feasibility of this therapeutic strategy for SCI.

2. Materials and Methods

2.1. Synthesis of furan-modified gellan gum hydrogel

Gellan gum (Sigma, USA) was first dissolved in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (100 mM, pH 5.5) at 37 °C. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Sigma, USA) and furfurylamine (Furan, Acros Organics, Belgium) were then added in a 4:1 molar ratio (relative to the –COOH groups in gellan gum) and stirred at 37 °C for 48 hours. The solution was then dialyzed (M_w cutoff 12-14kDaltons, Spectrum Labs, USA) alternately against distilled water and PBS (0.1M and pH 7.2) for 5 days. Finally, water was removed by lyophilization to obtain furan-modified gellan gum (furan-GG) as a white powder. ^1H -NMR spectra were used to analyze the degree of furan substitution. ^1H -NMR was recorded in D_2O on a

Varian Mercury-400 MHz NMR spectrometer (Palo Alto, USA). As a negative control, GG was incubated with furan in the absence of DMTMM.

2.2. *Synthesis of maleimide-modified GRGDS peptide*

Maleimide-modified GRGDS (mal-GRGDS) peptide was prepared by linear solid-phase synthesis (SPS) using standard Fmoc chemistry [31]. Fmoc-serine Wang resin (Anaspect, USA) with a loading capacity of 0.48 mmol/g was swollen in DMF (Sigma, USA) for 30 minutes. The solution was then drained and 20% of piperidine (Caledon, Canada) in DMF was added. After 30 minutes of incubation the resin was washed with DMF and tested for free amines using 1 % trinitrobenzene sulfonate, TNBS (TCI America, USA), in DMF. The first building block (Fmoc-asp-OH, 3.0 equivalents, all amino acids are from NovaBiochem, Germany) was pre-mixed with 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate, HCTU (3.0 equivalents, Anaspect, USA) in DMF for 15 min, then transferred to the SPS flask. Diisopropylethylamine, DIPEA (4.0 equivalents, Sigma, USA) in DMF, was then added and the flask was stirred for 24 hours. To verify if the coupling reaction had reached completion, the TNBS test was performed (negative test outcome). The Fmoc deprotection and coupling steps were repeated until 5 amino acid residues (GRGDS) were coupled to the resin. To conjugate the maleimide linker to the deprotected N-terminus of the peptide, 4 equivalents of maleimidopropionic acid (TCI America, USA) and 12 equivalents of diisopropylcarbodiimide (Sigma, USA) were pre-mixed in dichloromethane (Sigma, USA) for 45 minutes and then added to the SPS flask and stirred for 24 hours. The maleimide-modified GRGDS (mal-GRGDS) sequence was then cleaved from the resin using 95% trifluoroacetic acid (Caledon, Canada) in water.

The peptide was allowed to precipitate in cold diethyl ether for 30 minutes. Then, the precipitate was recovered by centrifugation. Finally, the product was purified by HPLC (Shimadzu, Japan) in a C18, 250 x 10 mm, 5 μ m, 100Å column. A mobile phase gradient from 5 % to 20 % (Acetonitrile (w/ 0.1 % TFA):ddH₂O (w/ 0.1 % TFA)) over 30 minutes was performed. A 90% yield was obtained and the product was confirmed by ¹H NMR and mass spectrometry (MS).

2.3. Immobilization of mal-GRGDS peptide on furan-GG hydrogel by Diels-Alder chemistry

Immobilization of maleimide-containing GRGDS (mal-GRGDS) to furan-modified gellan gum was performed by via Diels-Alder chemistry between the maleimide functional group of the peptide with the furan group of the gellan gum. Furan-GG was first dissolved in MES buffer (100 mM, pH 5.5) at 4 mg/ml and 37 °C and then incubated with mal-GRGDS in a 1:5 furan-maleimide molar ratio for 48h under constant stirring. The solution was then dialyzed (M_w cutoff 12-14kDaltons, Spectrum Labs, USA) alternately against distilled water and PBS (0.1M and pH 7.2) for 5 days. Finally, the water was removed by lyophilization to obtain GRGDS-modified Gellan Gum (GRGDS-GG) as a white powder. The amount of peptide immobilized on the hydrogel was calculated by amino acid analysis. In brief, this method involved acid hydrolysis of the peptide with 6 N HCl for 24 h, followed by derivatization with phenylisothiocyanate (PITC). The derivatized hydrolyzates were then quantified using reverse phase HPLC. As a negative control, mal-GRGDS was incubated with unmodified gellam gum.

2.4. *Cell isolation and culture*

Neural stem/progenitors cells were isolated from the subependymal region of the lateral ventricles in the forebrain of adult male Wistar rats as previously described [32]. Cells were grown in neurobasal medium (Invitrogen, Canada), with 2% of B27 neural supplement (Invitrogen), 1% of L-glutamine (Sigma), 1% of penicillin–streptomycin (Sigma), 20 ng/ml EGF (recombinant human EGF; Invitrogen), 20 ng/ml bFGF (recombinant human bFGF; Invitrogen) and 2 ng/ml heparin (Sigma). Cell number and viability were determined with a hemocytometer and the trypan blue exclusion test. Dissociated cells were plated in complete media and incubated in a humidified atmosphere at 37 °C with 5% of CO₂. Neurospheres were observed within 2–3 weeks, after which cells were passaged weekly.

Olfactory ensheathing cells were isolated from adult male Wistar rats as previously described [33]. Briefly, upon the olfactory bulb dissection all meninges were removed and the tissue was digested with 0,125% Collagenase (Sigma) for 20 min at 37 °C. The digested tissue was mechanically dissociated with a pipette and then filtrated through a 70 µm cell strainer (BD Falcon, USA). After centrifugation at 1000rpm for 10 min, cells were resuspended and plated in uncoated plates for 18h. A posterior change to new uncoated plates for 36h was made, as it is expected that most of the fibroblasts and astrocytes will attach in the first and second period, respectively. Finally, the cell suspensions were transferred to fibronectin treaded flasks (overnight coated with 1µg/ml fibronectin solution, Sigma, USA) and cultured in DMEM/F12 (Gibco) with 10% of FBS (Gibco) and 1% of antibiotic-antimycotic solution (Sigma) at 37 °C and 5% CO₂. OECs were enriched by the supplementation with Bovine Pituitary Extract (5µg/ml, Gibco) and Forskolin (2µg/ml, Sigma).

2.5. *Neural stem/progenitors cells culture on GRGDS-modified gellan gum*

Bioactivity of GRGDS-GG was assessed by the analysis of NSPCs growth, morphology and differentiation when cultured on the hydrogel for 2 and 7 days. NSPCs were mechanically dissociated into a single cell suspension and either seeded on the surface or encapsulated into GRGDS-GG hydrogel. The cell density was 2×10^4 cells/cm² and hydrogels were incubated in a humidified atmosphere at 37 °C and 5% of CO₂. Furan-modified GG was used as a control. Cell growth and morphology was evaluated following phalloidin/DAPI staining and differentiation following immunocytochemistry (ICC). Analysis was carried out using a Zeiss Observer Z1 microscope with a Yokogawa confocal scan unit; images were captured and processed using Volocity 4.3.2 software.

2.6. *Co-cultures between OECs and NSPCs*

To evaluate the potential synergistic or antagonistic effects between OECs and NSPCs, direct and transwell co-culture experiments were performed. OECs (1×10^5 cells/cm²) and NSPCs (4×10^4 cells/cm²), obtained as described in section 2.4, were seeded either simultaneously on the same fibronectin coated cover glass or in indirect contact with OECs cultured on the fibronectin coated cover glass and NSPCs on the transwell. Cells were allowed to grow in complete NSPCs culture medium (see section 2.4) for 24h and then cultured in the absence of the growth factors FGF and EGF. In the direct co-culture experiment, in order to clearly identify one cell population from another, OECs were labeled (according to manufacturer instructions) with a cell tracing reagent (C34554, Invitrogen) before seeding. After 7 days of incubation, cell growth and NSPCs

differentiation was assessed by ICC. Analysis was performed using an Olympus BX61 fluorescence microscope. OECs and NSPCs cultured alone were used as controls.

After these initial experiments, OECs and NSPCs were then encapsulated together in the GRGDS-GG hydrogel. Both cells were pre-labeled before encapsulation. A green tracer was used for OECs labeling (C34554, Invitrogen) and NSPCs were labeled with a far red tracer (C34553, Invitrogen). Both labeling protocols were performed according to the manufacturer instructions. Cell interactions and growth were then analyzed by confocal analyses after 7 days of culture.

2.7. Immunocytochemistry and phalloidin/DAPI staining

The following primary antibodies were used for the immunocytochemical studies: monoclonal rabbit anti- β -III tubulin (1:500, Chemicon, Canada) for neurons; monoclonal mouse anti-GFAP (1:100, Chemicon) for astrocytes; monoclonal mouse anti-O4 (1:200, R&D Systems, Canada) for oligodendrocytes; monoclonal mouse anti-Nestin (1:100, Millipore, USA) for progenitor cells; and polyclonal rabbit anti-p75 (1:100, Millipore) for OECs. For all immunohistochemical procedures, the appropriate controls were obtained by omission of the relevant primary antibody. Cells on the substrates were fixed with PBS solution containing 4% paraformaldehyde PFA for 20 mins (on glass) or 1 h (in the hydrogel) at room temperature and then washed with PBS. Next, cell membrane permeation (except for p75 and O4 antibodies) and blocking by treating cells with 0.3% Triton X-100 (Sigma, USA) and 10% of FBS solution at room temperature for 1 h, each specific primary antibody solution was added for 1h (on glass) or 12h (on hydrogel). After washing with 0.5% of FBS in PBS, the samples were exposed to the specific secondary antibody (Invitrogen) for 1h (on glass) or 5h (on

hydrogel) and then washed with 0.5% FBS. Finally, cell nuclei were counterstained with 1 $\mu\text{g/ml}$ DAPI (Invitrogen) for 1h.

For the phalloidin/DAPI staining, cells were fixed with 4% of PFA for 30 min at room temperature and then treated with 0.3% TritonX-100. After washing several times with PBS, 0.1 $\mu\text{g/ml}$ of phalloidin (Sigma) was added to the cells for 30 min. Finally, cell nuclei were counterstained with DAPI (1 $\mu\text{g/ml}$, Invitrogen) for 10 min.

2.8. *Statistical analysis*

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences among groups were assessed by two-way ANOVA followed by a Bonferroni post-hoc test (proliferation and differentiation analyses of NSPCs) and by t-student test (proliferation analyses of NSPCs and OECs in the co-cultures experiments). A p-value of ≤ 0.05 (95% confidence level) was set as the criteria for statistical significance. All data are presented as mean \pm standard deviation.

3. Results

3.1. *Synthesis and characterization of GRGDS-GG hydrogel*

Immobilization of the synthetic peptide (mal-GRGDS) to the gellan gum (GG) hydrogel was achieved in two synthetic steps (Fig 1a). First, the carboxylic acid groups of the glucuronic acid monosaccharide of GG were activated with DMT-MM, and then conjugated to furfurylamine to functionalize the GG with a furan. By ^1H -NMR analysis, the degree of furan substitution to GG was calculated to be 27% (Fig. 1b). This was calculated by comparing the ratio of the areas under the furan peaks at 6.26, 6.46 and 7.65 ppm to the methyl peak at 1.2 ppm (of the rhamnose monosaccharide of GG). To confirm that the furfurylamine was covalently bound to GG, and not simply adsorbed, a control reaction (in the absence of the coupling agent, DMT-MM) was similarly characterized, and analysis of this ^1H -NMR spectrum did not show any furan peaks (Fig. 1c). Second, the furan-GG hydrogel was reacted with maleimide-modified GRGDS peptide to yield GG-GRGDS hydrogel. After excessive dialysis to remove unbound peptide, quantification by amino acid analysis of the immobilized peptide was calculated to be 304.0 nmol of GRGDS peptide per mg of GG (Fig. 1d). A control reaction (using unmodified GG) was also performed to determine the amount of unbound peptide adsorbed on the hydrogel. In the absence of furan substitution to GG, only 5.3 nmol of GRGDS/mg of GG (Fig. 1e) was detected. Thus, approximately 300 nmol/mg of GRGDS peptide was covalently bound to the GG by the Diels-Alder [4+2] cycloaddition.

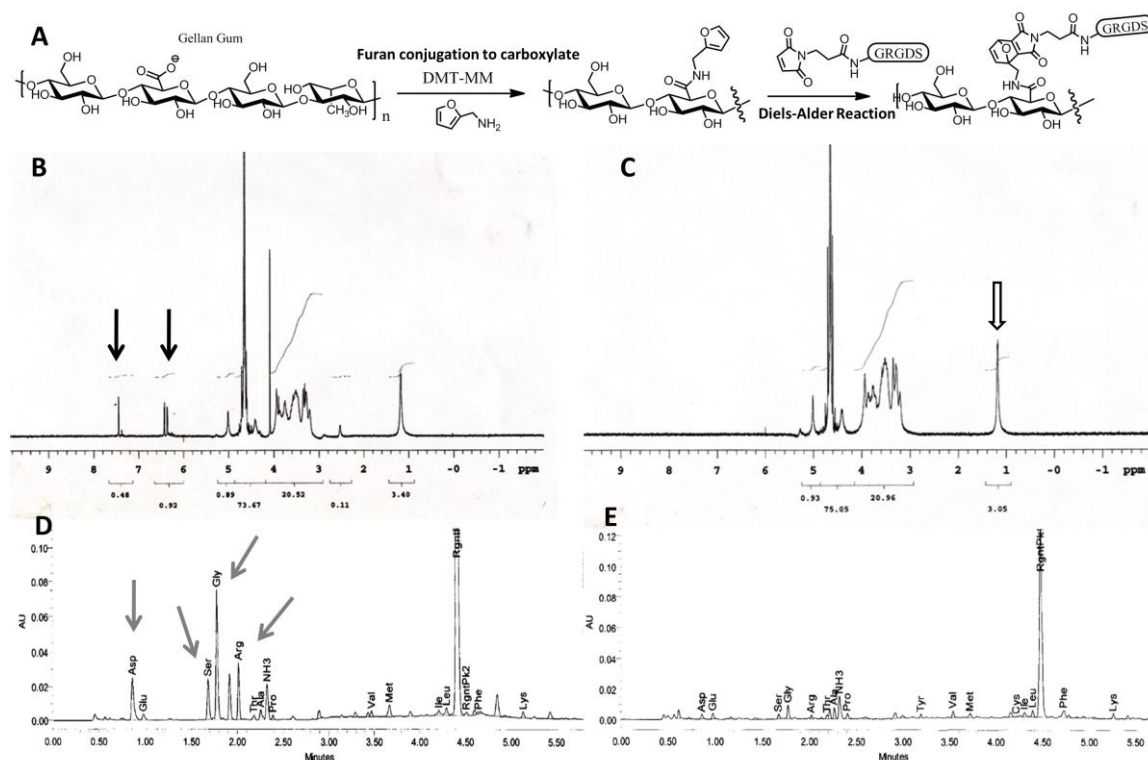


Figure VI.1. Immobilization of the GRGDS peptide into the Gellan Gum hydrogel. (A) Schematic representation of the Diels-Alder reaction (furan-maleimide conjugation) used for the GG modification. When DMT-MM and furfurylamine were incubated with the GG, a furan-modified gel with 27% substitution was synthesized. (B) ¹H-NMR spectrum of furan-GG. The degree of substitution was calculated by comparing the ratio of the areas under the proton peaks at 6.26, 6.46 and 7.65 ppm (black arrows point to furan protons) to the peak at 1.2 ppm (white arrow point to methyl group of GG). (C) ¹H-NMR spectrum of GG, after incubation with furfurylamine in the absence of the coupling reagent DMT-MM. Furan peaks were not detected in the GG spectrum (D) By reverse phase HPLC, the amino acid analyses revealed that when furan-GG and mal-GRGDS were incubated together it was possible to obtain approximately 300 nmol GRGDS/mg of GG (gray arrows point to each amino acid). (E) In contrast, only 5.3 nmol of GRGDS peptide were detected in the control reaction between maleimide-modified GRGDS and unmodified GG.

3.2. Biological effect of GRGDS-GG on NSPCs

NSPCs were either seeded on the surface or encapsulated within GRGDS-GG vs. unmodified GG hydrogels. Cell proliferation, morphology and differentiation were assessed after 2 and 7 days of culture. The results revealed pronounced differences in

NSPC behavior when cultured in the peptide-modified hydrogel relative to the unmodified hydrogel. In the presence of GRGDS, the cells were able to migrate and successfully expand throughout the hydrogel whereas in the absence of GRGDS, NSPCs interacted preferentially with each other, forming cell aggregates or neurospheres (Fig. 2). Visible cytoplasmatic extensions were observed in the GRGDS-GG, either on the surface (Fig. 2a) or inside the gel (Fig. 2b), but not on the unmodified GG.

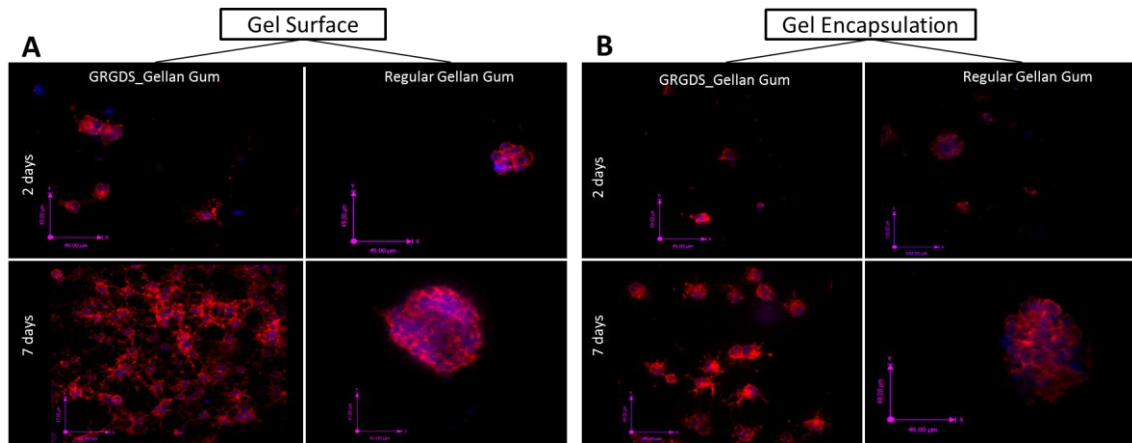


Figure VI.2. Morphology and dispersion of NSPCs on the GG-GRGDS. Confocal analyses revealed substantial differences in NSPC morphology when cultured either (A) on the surface or (B) encapsulated within the GG-GRGDS vs. unmodified GG gel. Cell spreading and visible cytoplasmatic extensions were only observed in the GG-GRGDS. In the unmodified GG, NSPCs proliferated as neurospheres. The cytoplasm was stained with the anti-F-actin / phalloidin (red) and nuclei counterstained with DAPI (blue).

Proliferation and morphological analyses revealed that in the absence of peptides, the cells were only able to proliferate as neurospheres. In the GRGDS-GG hydrogel cells proliferated as single cells. The number of single cells found either encapsulated within or on the gel surface was significantly higher in the GRGDS-modified hydrogel than the unmodified GG gel (Fig. 3a and 3b). For example, after 7 days of culture, we observed an average of 60 ± 29 single cells/mm² on the GRGDS-GG gel surface whereas only $4 \pm$

2 single cells/mm² on the GG surface. A similar trend was observed with encapsulated cells. The number of single cells in the GRGDS-GG hydrogel of 23 ± 7 single cells/mm² was significantly higher than that in the GG hydrogel of 2 ± 2 . Interestingly, while GRGDS modification influenced the number of single cells present, it did not affect the differentiation profile, which was largely the same (Fig 3c). After 7 days, the majority of NSPCs on GRGDS-GG vs. GG hydrogels, respectively, were O4 positive oligodendrocytes $72 \pm 11\%$ vs $64 \pm 30\%$, with relatively little differentiation into astrocytes $7 \pm 12\%$ vs $0 \pm 0\%$ and no differentiation into neurons. Moreover, as judged by the nestin expression, some NSPCs remained as progenitors $32 \pm 16\%$ vs $36 \pm 19\%$.

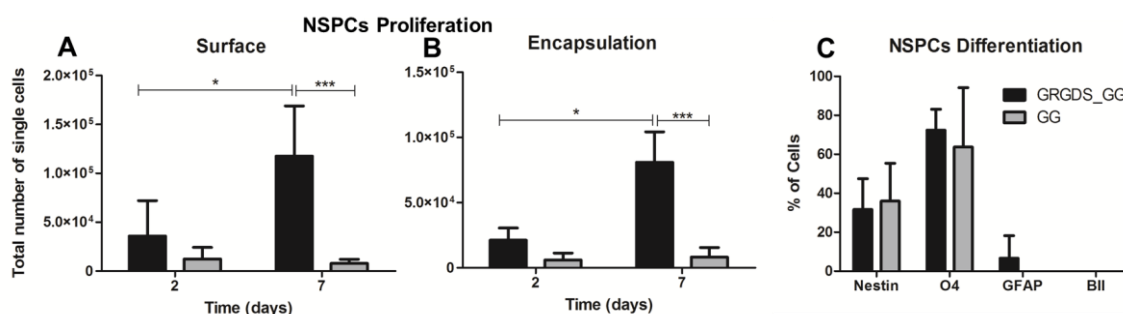


Figure VI.3. Bioactivity of the GRGDS-GG hydrogel. Proliferation analyses of NSPCs cultured either (A) on the surface of or (B) in the gel of the GRGDS-GG and regular GG showed that on the seventh day a significantly higher number of single cells were found in the GRGDS-GG when comparing with the regular GG gel. Moreover, only in the GRGDS-GG a significant increase was observed from day 2 to day 7. Finally, immunocytochemistry revealed that the GRGDS sequence did not significantly influence the differentiation profile of the NSPCs (C). Values are shown as mean \pm standard deviation (n=3 samples of 2×10^4 cells/sample, *p<0.05; ***p<0.001)

3.3. *Co-culture of OECs and NSPCs*

The interaction of OECs and NSPCs were analyzed after 7 days in culture for cell proliferation, and differentiation as a function of direct or indirect (i.e. same media, but not contact through use of a transwell) contact. Interestingly, the differentiation profile of NSPCs was not altered by the presence of the OECs (Fig. 4a); however, NSPCs proliferated significantly more when co-cultured with OECs vs. alone. There was a 2.3 fold increase in the number of NSPCs when co-cultured in direct contact with OECs vs. NSPCs alone (Fig 4b) and a 1.8-fold increase in the number of NSPCs when co-cultured indirectly in transwells with OECs vs. NSPC monoculture (Fig 4c). These results suggest that OECs secrete factors that enhance NSPCs proliferation and that the cells do not need to be in contact in order for this affect to be observed. Moreover, analysis of cell dispersion shows that OECs and NSPCs highly interact with each other. Unlike the proliferative affect that the OECs had on the NSPCs, the converse was not true: the total number of OECs was not significantly affected by co-culture with NSPCs either in direct contact (Fig 4d) or indirect, transwell (Fig 4e) contact. Notwithstanding that OECs do not have to be in direct contact with NSPCs to influence their proliferation, immunocytochemistry illustrates that OECs are closely associated with NSPCs when co-cultured together (Fig 5). By pre-labelling OECs green with CFSE34554 and counterstaining NSPCs with either nestin (red for progenitors) or O4 (red for oligodendrocytes), the two cell types appeared to be closely associated with each other. These data suggest that OECs may be able to provide a guidance pathway for NSPC growth.

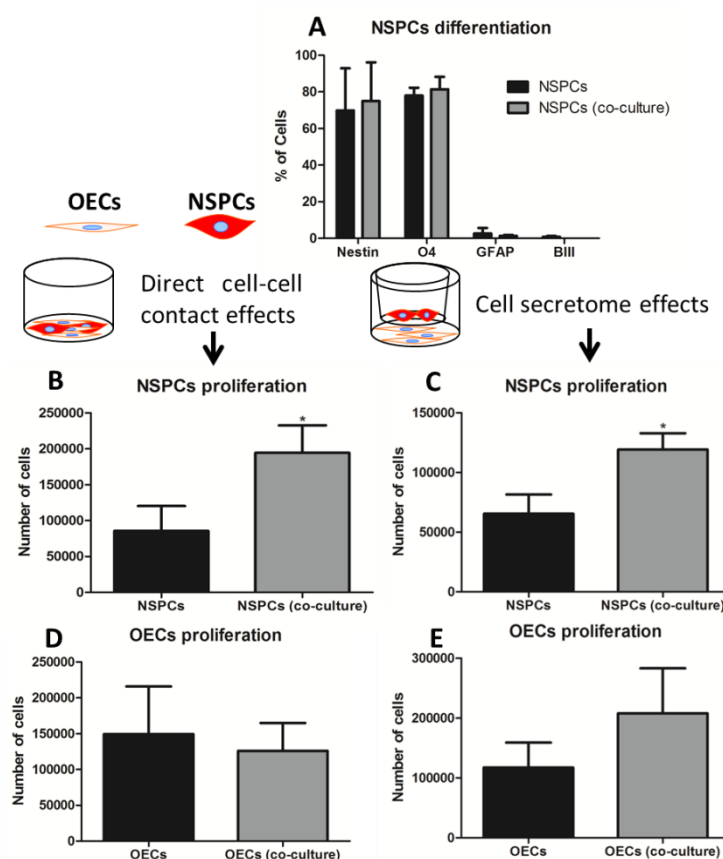


Figure VI.4. OECs and NSPCs proliferation in transwells and direct contact co-culture. (A) The differentiation profile of NSPCs was not affected by the presence of OECs. Proliferation analysis has shown that the total number of NSPCs is significantly higher when in culture with OECs than when cultured alone. This occurred both in the (B) direct cell-cell contact co-cultures and in the (C) indirect transwell co-cultures. OECs proliferation was not significantly affected by the presence of NSPCs, either in (D) direct contact or in the (E) indirect transwell co-culture. Values are shown as mean \pm standard deviation (n=3 independent studies of a minimum of 4000 cells counted per study, *p<0.05).

3.4. Co-culture of OECs and NSPCs in the GRGDS-GG hydrogel

To gain greater insight into the interactions of OECs and NSPCs, they were co-cultured together in the 3D gellan gum hydrogel modified with the cell-adhesive peptide, GRGDS (GRGDS-GG). To facilitate identification, the OECs were pre-labeled green with CFSE 34554 and the NSPCs red with DDAO-SE 34553 prior to encapsulation in

the GRGDS-GG hydrogel. As was observed in the 2D co-culture study, the total number of NSPCs was significantly higher when co-cultured with OECs than when cultured alone (Fig. 6a). Similar to the 2D data presented in Fig 4, the OEC proliferation was unaffected by the presence of NSPCs (Fig. 6b).

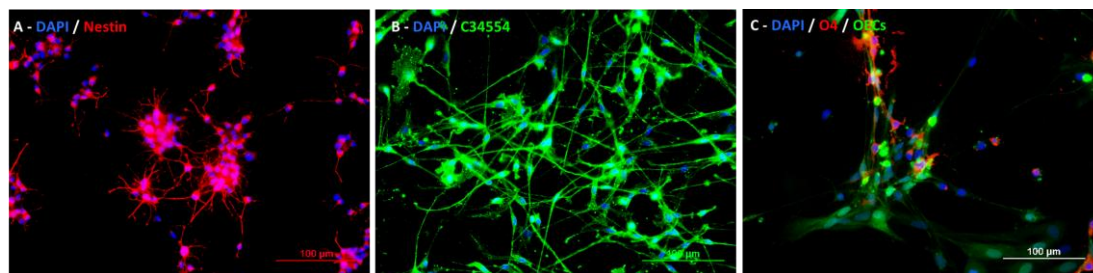


Figure VI.5. OECs and NSPCs interact with each other during co-culture. (A) Monocultures of NSPCs and (B) monocultures OECs served as controls. (C) OECs (green, CFSE 34554) and NSPCs (red, O4) appear to be closely associated when the cells are cultured together in direct contact. NSPCs were identified by immunocytochemistry and nuclei are counterstained with DAPI (blue).

4. Discussion

Gellan gum (GG) is a natural biomaterial that has shown promise for tissue regeneration. It is a thermo-reversible gel that was previously shown to be cytocompatible with adipose stem cells, rabbit articular chondrocytes and immortalized oligodendrocytes [20, 34]. Until recently, most of the research on GG modification was focused on controlling its mechanical properties through gel network formation [21, 35]. To the best of our knowledge, there is no literature describing the chemical modification of GG with ECM-derived peptides, even though the cellular microenvironment is defined, in part, by its chemical nature. In order to create a hydrogel which mimics the chemical nature of the ECM, bioactive peptides were conjugated to GG, thereby providing a more suitable environment for encapsulated cells to survive and migrate.

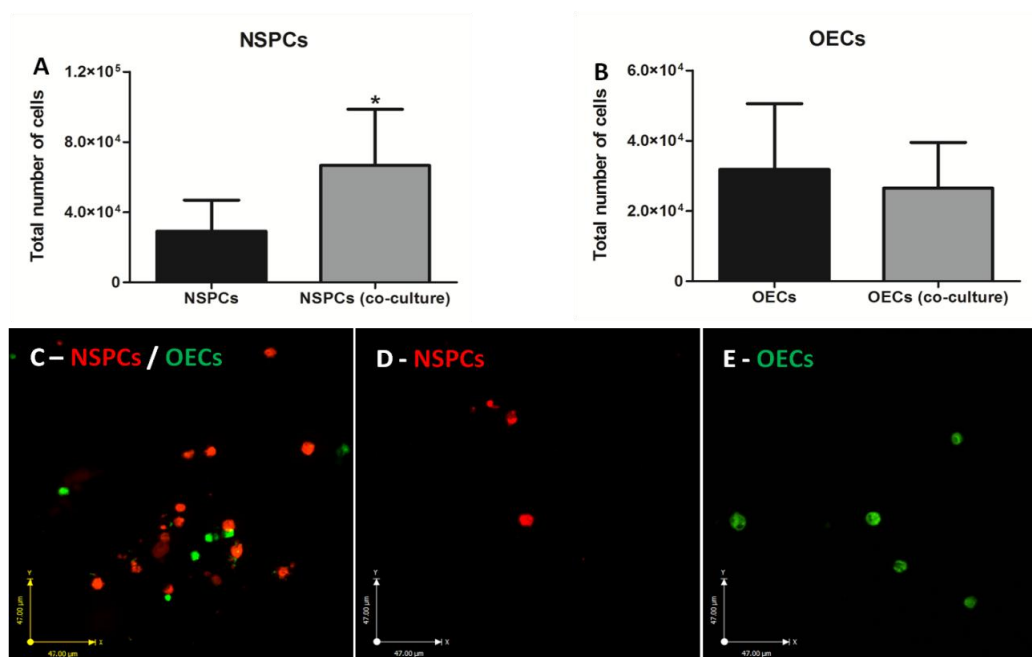


Figure VI.6. Co-culture between OECs and NSPCs in the GRGDS-GG hydrogel. (A) After counting the cells was possible to observe that the number of NSPCs was significantly higher when co-cultured with OECs than when cultured alone. (B) In contrast, OEC proliferation was unaffected by the presence of NSPCs in culture. (C) Co-cultures of NSPCs (red, DDAO-SE 34553) and OECs (green, CFSE 34554) suggested some interaction between the two cell types as observed by confocal microscopy. (D) NSPCs and (E) OECs were cultured alone and served as controls. Values are shown as mean \pm standard deviation (n=3 independent experiments of 200 cells counted per experiment, *p<0.05)

The peptide sequence GRGDS is a ubiquitous cell-adhesive peptide, which has been shown to enhance cell-biomaterial interactions, support cell survival and influence cell morphology [22, 36]. In this work we show that it is possible to immobilize the GRGDS peptide to GG. By taking advantage of the Diels-Alder click cycloaddition reaction between chemically modified GG-furan and maleimide-GRGDS, GG-GRGDS was synthesized in aqueous conditions.

Immobilization of GRGDS to GG had a profound effect on NSPC morphology, distinct from that observed on NSPCs in unmodified GG alone, demonstrating the importance of GRGDS for cell-GG interaction. Importantly, these morphological differences were

observed both within the 3D hydrogels and on their surfaces. Unlike most studies that focus only on cells cultured on surfaces, herein we cultured cells both on the hydrogel surface and encapsulated within [36, 37]. Given that the ultimate goal involves cell transplantation via an injectable hydrogel, it was reassuring that the cell morphology in 3D mirrored that in 2D. The NSPCs were able to adhere to and extend processes within the GG-GRGDS hydrogel, yet their differentiation profile was not affected by the presence of GRGDS. This reflects the cell-adhesive property ascribed to GRGDS and not a differentiation property. To achieve preferential differentiation to a given phenotype, GG would typically require further modification with growth factors, such as PDGF-AA for oligodendrocytes [38] or interferon-gamma for neurons [39].

The co-culture experiments of NSPCs and OECs, in direct contact or in the transwell, suggested that diffusible factors are responsible for the effects observed. OECs have been shown to secrete several neurotrophic factors including: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) [13], basic fibroblast growth factor (FGF2) and neurotrophin-3 (NT-3) [40]. This rich profile of secreted neurotrophins from OECs may explain the proliferation of NSPCs that we observed in our co-culture studies. For example, NGF and FGF promote NSPC proliferation [41, 42]; and NT3 enhances their survival [43]. The combination of both mechanisms, survival and proliferation, may explain the increased number of NSPCs in all of the co-culture studies.

Other than this study, very little is known about the effects of OECs on the behavior of NSPCs. Interestingly, Cao *et al* [33], also observed that OECs promoted NSPC proliferation, yet they also observed increased neurogenesis and oligodendrogenesis of NSPCs cultured with OECs. Differences in the co-culture experiments may account for this discrepancy. Cao *et al* harvested the NSPCs from newborn mice, whereas we

obtained NSPCs from adult rats; moreover, they co-cultured the cells for only 3 days in DMEM with F12 and N2 supplements whereas we co-cultured the cells for 7 days in neurobasal medium with B27 supplements.

The immunocytochemistry figures suggest that OECs and NSPCs interact with each other. This interaction may be attributed to OECs expressing cell adhesive molecules, such as N-CAM and L1 [44], and NSPCs expressing the corresponding receptors [45]. The cellular interactions may suggest a role for co-transplantation of OECs and NSPCs.

Cell transplantation has been pursued for several years for SCI repair. Both OECs and NSPCs have been investigated independently, each with some success. Notably, biomaterial scaffolds have been shown to enhance cell survival after transplantation. For example, Cummings *et al* [46] showed that transplanting human NSPCs in SCI mice led to some locomotor recovery and remyelination. Johnson *et al* [47] demonstrated that implantation of fibrin hydrogels containing neural progenitor cells resulted in some functional recovery in SCI rats. Ballios *et al* demonstrated greater cell survival after transplantation in a hyaluronan/methylcellulose hydrogel compared to saline [48]. OECs support and guide axon elongation in their native olfactory system and have shown promise after spinal cord injury as well [49, 50]. OECs transplanted immediately or up to 2 months after SCI in rats resulted in functional recovery and/or tissue regeneration [51, 52]. Significantly, OECs have been tested in three separate clinical trials [30, 53, 54]; however, only very modest (if any) motor improvements were observed. Despite the promise for cell therapy, with OECs or NSPCs, the functional recovery has been modest, underlining the need for innovative strategies, such as the combined transplantation of OECs and NSPCs in a GG-GRGDS hydrogels. The simplicity and broad applicability of the Diels-Alder click chemistry can be easily

extended to other biomolecules to further promote NSPC differentiation for greater integration with the host tissue. This combination strategy could be powerful, with OECs and gellan gum providing a cellular pathway on which NSPCs could differentiate, thereby replacing the damaged tissue and achieving greater functional repair.

5. Conclusions

Using well-established Diels-Alder click-chemistry, we immobilized the GRGDS fibronectin-derived peptide to gellan gum hydrogels, which promoted greater adhesion and proliferation of neural stem/progenitor cells than gellan gum controls. Moreover, NSPCs co-cultured with olfactory ensheathing glia showed greater survival and outgrowth than NSPCs cultured alone. These results suggest that the combined use of NSPCs and OECs with bioengineered GG-GRGDS hydrogels may be beneficial in regenerative medicine cell transplantation strategies to promote repair after spinal cord injury.

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References

- [1] Bracken MB. Methylprednisolone and Acute Spinal Cord Injury: An Update of the Randomized Evidence. *Spine*. 2001;26:S47-S54.
- [2] Hurlbert RJ. Methylprednisolone for acute spinal cord injury: an inappropriate standard of care. *Journal of Neurosurgery*. 2000;93:1-7.
- [3] Casha S. Minocycline and Perfusion Pressure Augmentation in Acute Spinal Cord Injury, NCT00559494, <http://clinicaltrials.gov/ct2/show/NCT00559494?term=NCT00559494&rank=1>. Clinicaltrials.gov; 2008.
- [4] Novartis. Acute Safety, Tolerability, Feasibility and Pharmacokinetics of Intrath. Administered ATI355 in Patients With Acute SCI, NCT00406016, <http://clinicaltrials.gov/ct2/show/NCT00406016?term=NCT00406016&rank=1>. Clinicaltrials.gov; 2011.
- [5] Geron. Safety Study of GRNOPC1 in Spinal Cord Injury, NCT01217008, <http://clinicaltrials.gov/ct2/show/NCT01217008?term=NCT01217008&rank=1>. Clinicaltrials.gov; 2012.
- [6] Lu J, Féron F, Mackay-Sim A, Waite PME. Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord. *Brain*. 2002;125:14-21.
- [7] Li Y, Field PM, Raisman G. Repair of Adult Rat Corticospinal Tract by Transplants of Olfactory Ensheathing Cells. *Science*. 1997;277:2000-2.
- [8] Kim H, Zahir T, Tator CH, Shoichet MS. Effects of Dibutyl Cyclic-AMP on Survival and Neuronal Differentiation of Neural Stem/Progenitor Cells Transplanted into Spinal Cord Injured Rats. *PLoS ONE*. 2011;6:e21744.
- [9] Pal R, Gopinath C, Rao NM, Banerjee P, Krishnamoorthy V, Venkataramana NK, et al. Functional recovery after transplantation of bone marrow-derived human

mesenchymal stromal cells in a rat model of spinal cord injury. *Cytherapy*. 2010;12:792-806.

[10] Arboleda D, Forostyak S, Jendelova P, Marekova D, Amemori T, Pivonkova H, et al. Transplantation of Predifferentiated Adipose-Derived Stromal Cells for the Treatment of Spinal Cord Injury. *Cellular and Molecular Neurobiology*. 2011;1-10.

[11] Doucette R. Glial influences on axonal growth in the primary olfactory system. *Glia*. 1990;3:433-49.

[12] Ramón-Cueto A, Nieto-Sampedro M. Regeneration into the Spinal Cord of Transected Dorsal Root Axons Is Promoted by Ensheathing Glia Transplants. *Experimental Neurology*. 1994;127:232-44.

[13] Woodhall E, West AK, Chuah MI. Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors. *Molecular Brain Research*. 2001;88:203-13.

[14] Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Morshead CM, Fehlings MG. Delayed Transplantation of Adult Neural Precursor Cells Promotes Remyelination and Functional Neurological Recovery after Spinal Cord Injury. *The Journal of Neuroscience*. 2006;26:3377-89.

[15] McDonald JW, Liu X-Z, Qu Y, Liu S, Mickey SK, Turetsky D, et al. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med*. 1999;5:1410-2.

[16] Robey TE, Saiget MK, Reinecke H, Murry CE. Systems approaches to preventing transplanted cell death in cardiac repair. *Journal of Molecular and Cellular Cardiology*. 2008;45:567-81.

[17] Zvibel I, Smets F, Soriano H. Anoikis: Roadblock to Cell Transplantation? *Cell Transplantation*. 2002;11:621-30.

[18] Moorhouse R, Colegrove G T, Sandford P A, Baird J K, Kang K S. PS-60: A New Gel-Forming Polysaccharide. *Solution Properties of Polysaccharides: AMERICAN CHEMICAL SOCIETY*; 1981. p. 111-24.

[19] Kubo W, Miyazaki S, Attwood D. Oral sustained delivery of paracetamol from in situ-gelling gellan and sodium alginate formulations. *International Journal of Pharmaceutics*. 2003;258:55-64.

[20] Oliveira JT, Gardel LS, Rada T, Martins L, Gomes ME, Reis RL. Injectable gellan gum hydrogels with autologous cells for the treatment of rabbit articular cartilage defects. *Journal of Orthopaedic Research*. 2010;28:1193-9.

- [21] Silva-Correia J, Oliveira JM, Caridade SG, Oliveira JT, Sousa RA, Mano JF, et al. Gellan gum-based hydrogels for intervertebral disc tissue-engineering applications. *Journal of Tissue Engineering and Regenerative Medicine*. 2011;5:e97-e107.
- [22] Luo Y, Shoichet MS. A photolabile hydrogel for guided three-dimensional cell growth and migration. *Nat Mater*. 2004;3:249-53.
- [23] Tong YW, Shoichet MS. Enhancing the neuronal interaction on fluoropolymer surfaces with mixed peptides or spacer group linkers. *Biomaterials*. 2001;22:1029-34.
- [24] Yu TT, Shoichet MS. Guided cell adhesion and outgrowth in peptide-modified channels for neural tissue engineering. *Biomaterials*. 2005;26:1507-14.
- [25] Adams JC, Watt FM. Regulation of development and differentiation by the extracellular matrix. *Development*. 1993;117:1183-98.
- [26] Schwarz M, Mitchell M, Emerson D. Reconstituted basement membrane enhances neurite outgrowth in PC12 cells induced by nerve growth factor. *Cell Growth Differ*. 1990;1:313-8.
- [27] Cooke MJ, Vulic K, Shoichet MS. Design of biomaterials to enhance stem cell survival when transplanted into the damaged central nervous system. *Soft Matter*. 2010;6:4988-98.
- [28] Kim H, Cooke MJ, Shoichet MS. Creating permissive microenvironments for stem cell transplantation into the central nervous system. *Trends in Biotechnology*. 2012;30:55-63.
- [29] Ayuso-Sacido A, Roy NS, Schwartz TH, Greenfield JP, Boockvar JA. Long-term expansion of adult human brain subventricular zone precursors. *Neurosurgery*. 2008;62:223-9.
- [30] Feron F, Perry C, Cochrane J, Licina P, Nowitzke A, Urquhart S, et al. Autologous olfactory ensheathing cell transplantation in human spinal cord injury. *Brain*. 2005;128:2951-60.
- [31] Wellings DA, Atherton E. Standard Fmoc protocols. *Solid-Phase Peptide Synthesis*. 1997;289:44-67.
- [32] Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, et al. Neural stem cells in the adult mammalian forebrain: A relatively quiescent subpopulation of subependymal cells. *Neuron*. 1994;13:1071-82.
- [33] Cao L, Mu L, Qiu Y, Su Z, Zhu Y, Gao L, et al. Diffusible, membrane-bound, and extracellular matrix factors from olfactory ensheathing cells have different effects on

the self-renewing and differentiating properties of neural stem cells. *Brain Research*. 2010;1359:56-66.

[34] Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Pedro AJ, Leite-Almeida H, et al. Development and Characterization of a Novel Hybrid Tissue Engineering Based Scaffold for Spinal Cord Injury Repair. *Tissue Engineering Part A*. 2010;16:45-54.

[35] Coutinho DF, Sant SV, Shin H, Oliveira JT, Gomes ME, Neves NM, et al. Modified Gellan Gum hydrogels with tunable physical and mechanical properties. *Biomaterials*. 2010;31:7494-502.

[36] Lévesque SG, Shoichet MS. Synthesis of cell-adhesive dextran hydrogels and macroporous scaffolds. *Biomaterials*. 2006;27:5277-85.

[37] Fink H, Ahrenstedt L, Bodin A, Brumer H, Gatenholm P, Krettek A, et al. Bacterial cellulose modified with xyloglucan bearing the adhesion peptide RGD promotes endothelial cell adhesion and metabolism—a promising modification for vascular grafts. *Journal of Tissue Engineering and Regenerative Medicine*. 2011;5:454-63.

[38] Aizawa Y, Wylie R, Shoichet M. Endothelial Cell Guidance in 3D Patterned Scaffolds. *Advanced Materials*. 2010;22:4831-5.

[39] Leipzig ND, Wylie RG, Kim H, Shoichet MS. Differentiation of neural stem cells in three-dimensional growth factor-immobilized chitosan hydrogel scaffolds. *Biomaterials*. 2011;32:57-64.

[40] Mackay-Sim A, Chuah MI. Neurotrophic factors in the primary olfactory pathway. *Progress in Neurobiology*. 2000;62:527-59.

[41] Cattaneo E, McKay R. Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature*. 1990;347:762-5.

[42] Tropepe V, Sibilio M, Ciruna BG, Rossant J, Wagner EF, van der Kooy D. Distinct Neural Stem Cells Proliferate in Response to EGF and FGF in the Developing Mouse Telencephalon. *Developmental Biology*. 1999;208:166-88.

[43] Caldwell M, He X, Wilkie N, Pollack S, Marshall G, Wafford K, et al. Growth factors regulate the survival and fate of cells derived from human neurospheres. *Nature Biotechnology*. 2001;19:475–9.

[44] Steinke A, Meier-Stiegen S, Drenckhahn D, Asan E. Molecular composition of tight and adherens junctions in the rat olfactory epithelium and fila. *Histochemistry and Cell Biology*. 2008;130:339-61.

- [45] Gascon E, Vutskits L, Kiss JZ. The Role of PSA-NCAM in Adult Neurogenesis. Structure and Function of the Neural Cell Adhesion Molecule NCAM. In: Berezin V, editor. Structure and Function of the Neural Cell Adhesion Molecule NCAM: Springer New York; 2010. p. 127-36.
- [46] Cummings BJ, Uchida N, Tamaki SJ, Salazar DL, Hooshmand M, Summers R, et al. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:14069-74.
- [47] Johnson PJ, Tatara A, McCreedy DA, Shiu A, Sakiyama-Elbert SE. Tissue-engineered fibrin scaffolds containing neural progenitors enhance functional recovery in a subacute model of SCI. *Soft Matter*. 2010;6:5127-37.
- [48] Ballios BG, Cooke MJ, van der Kooy D, Shoichet MS. A hydrogel-based stem cell delivery system to treat retinal degenerative diseases. *Biomaterials*. 2010;31:2555-64.
- [49] Ramón-Cueto A, Avila J. Olfactory ensheathing glia: properties and function. *Brain Research Bulletin*. 1998;46:175-87.
- [50] Kubasak MD, Jindrich DL, Zhong H, Takeoka A, McFarland KC, Muñoz-Quiles C, et al. OEG implantation and step training enhance hindlimb-stepping ability in adult spinal transected rats. *Brain*. 2008;131:264-76.
- [51] Li Y, Decherchi P, Raisman G. Transplantation of Olfactory Ensheathing Cells into Spinal Cord Lesions Restores Breathing and Climbing. *The Journal of Neuroscience*. 2003;23:727-31.
- [52] Keyvan-Fouladi N, Raisman G, Li Y. Functional Repair of the Corticospinal Tract by Delayed Transplantation of Olfactory Ensheathing Cells in Adult Rats. *The Journal of Neuroscience*. 2003;23:9428-34.
- [53] Lima C, Pratas-Vital J, Escada P, Hasse-Ferreira A, Capucho C, Peduzzi JD. Olfactory mucosa autografts in human spinal cord injury: A pilot clinical study. *Journal of Spinal Cord Medicine*. 2006;29:191-203.
- [54] Dobkin BH, Curt A, Guest J. Cellular Transplants in China: Observational Study from the Largest Human Experiment in Chronic Spinal Cord Injury. *Neurorehabilitation and Neural Repair*. 2006;20:5-13.

CHAPTER VII

Combining Adult Stem Cells and Olfactory Ensheathing Cells: The Secretome Effect

Chapter VII.

Combining Adult Stem Cells and Olfactory Ensheathing Cells: The Secretome Effect

Abstract

Adult stem cells derived from adipose (ASCs), bone marrow (bmMSCs) and umbilical cord (HUCPVCs) tissue are been widely tested for regenerative applications, such as bone regeneration. Moreover, olfactory ensheathing cells (OECs) show promise in promoting spinal cord injury (SCI) regeneration. Our group recently proposed the use of a hybrid scaffold targeting both vertebral bone repair and SCI regeneration. In this concept, mesenchymal stem cells and OECs should be in close contact to be influenced by the factors that both secrete. For this reason, herein we studied the effects of the OEC secretome on the metabolic activity and proliferation of ASCs, bmMSCs and HUCPVCs. The stem cells' secretome effects on metabolic activity and proliferation of the OECs were also considered. In co-cultures of OECs with ASCs, bmMSCs or HUCPVCs the metabolic activity/viability, proliferation and total cell numbers were measured after 2 and 7 days of culture. The results demonstrated that the secretome of OECs has a positive effect on the metabolic activity and proliferation of mesenchymal stem cells from different origins, especially on ASCs. Furthermore, in general the stem cells' secretome also had a positive effect on the OECs behavior, particularly when ASCs were in co-culture with OECs. These results suggest that the most suitable combination of cells to be used in our hybrid scaffold is the OECs with the ASCs. Finally, this work adds important knowledge to the cell therapy field, bringing new information about paracrine interactions between OECs and distinct mesenchymal stem.

***These chapter is based on the following publication:**

Silva NA, Gimble JM, Sousa N, Reis RL, Salgado AJ, Combining Adult Stem Cells and Olfactory Ensheathing Cells: The Secretome Effect, Submitted.

1. Introduction

Transplantation of cells with regenerative capabilities holds great promise for the treatment of several diseases. However, the properties of the tissue into which the cells are to be transplanted, as well as the intrinsic properties of the transplanted cells will significantly influence the success of the therapy. In spinal cord injury (SCI) the host environment is particularly important. For instance, after SCI an environment of necrosis, edema, inflammation and degeneration emerge [1]. This unfavorable host environment will influence the ability of the transplanted cells to engraft, proliferate, differentiate and thus to contribute to the repair of the damaged tissue. In order to improve cell survival, several authors are proposing the use of hydrogels as vehicles for cell transplantation [2-4]. For example, Johnson *et al.* reported that fibrin scaffolds can enhance survival of neural stem/progenitors cells (NSPCs) in a sub-acute model of SCI [3]. Work from the Shoichet lab shows that a combination of cyclic-AMP, fibrin and chitosan channels greatly enhance the survival of NSPCs after transplantation in SCI rats [4]. Our group recently proposed the use of a hybrid tubular scaffold that comprise a rigid layer (composed by a blend of starch with polycaprolactone - SPCL), surrounding the hydrogel gellan gum [5]. In this concept the SPCL tubular structure assures mechanical stability to the entire construct, namely by establishing a connection to the adjacent vertebral bone [6], while the gellan gum hydrogel is aimed as a cell encapsulation system to support axonal regeneration in the injured spinal cord. In order to improve bone repair, mesenchymal stem cells, such as adipose derived adult stem cells (ASCs), human umbilical cord perivascular cells (HUCPVCs) or bone marrow mesenchymal stem cells (bmMSCs), can be seeded on the SPCL layer. Previous studies have demonstrated that these cells have the capability to undergo osteogenic differentiation and secrete extracellular matrix (ECM) rich in calcium phosphates (ECM

typically found in bone tissues) [7-9]. Alternatively, cells such as olfactory ensheathing cells (OECs), that are known to support and guide olfactory axons, secrete several neurotrophic factors and grow through the glial scar upon transplantation [10-12], are suitable candidates to be encapsulated in the hydrogel phase aimed to foster axonal regeneration. In this sense, our therapeutic approach puts in close contact both the mesenchymal stem cells and the OECs, allowing the secreted factors by these cells to diffuse and interact with each other (see schematic representation on Figure 1). For this reason, in this work we studied the interactions of the OECs secretome on the proliferation, metabolic activity and differentiation of ASCs, HUCPVCs and bmMSCs, as well as the effects of the stem cells secretome on OECs behavior. In this sense, we expect to find the best suitable combination to be used in our hybrid scaffold.

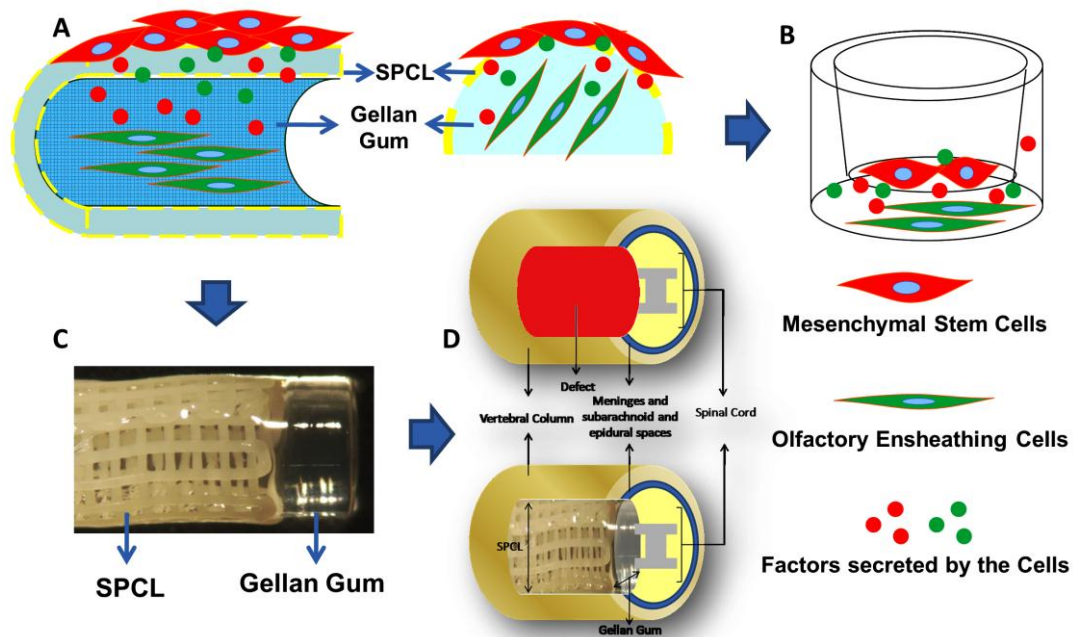


Figure VII.1. Rationality and schematic representation of the study. (A) Both adult stem cells (red cells seeded on the outer layer of the hybrid scaffolds), and OECs (green cells encapsulated on the hydrogel) secreted factors that can affect the biological behavior of each other. (B) Transwell system used to study the effects of cell secretome. (C) Hybrid scaffold developed and previously characterized [5]. (D) Schematic representation of the scaffold implantation, with the SPCL layer at vertebra bone level and the hydrogel in the nervous tissue level.

2. Materials and Methods

2.1. Cell Culture

To evaluate the potential positive or negative effects of the cell secretome of OECs and adult stem cells, transwell co-culture experiments were performed. OECs were seeded (1×10^5 cells/cm²) on poly-D-lysine cover glass and adult stem cells were seeded (4×10^4 cells/cm²) on the transwell. Cell proliferation, metabolic activity and differentiation were assessed after 2 and 7 days in culture. Cells were obtained as described below:

2.1.1. Olfactory Ensheathing Cells

Rat OECs were isolated as previously described [13]. Briefly, upon olfactory bulb collection, all meninges were removed and the tissue was digested with 0,125% Collagenase (Sigma) for 20 min at 37 °C. The digested tissue was mechanically dissociated with a pipette and then filtrated through a 70 µm cell strainer (BD Falcon, USA). After centrifugation at 1000rpm for 10 min, cells were resuspended and plated in uncoated wells for 18h. A posterior change to new uncoated wells for 36h was made, as it is expected that most of the fibroblasts and astrocytes will attach in the first and second period, respectively. Finally, the cell suspensions were transferred to poly-D-lysine treated flasks and cultured in DMEM/F12 (Gibco) with 10% of FBS (Gibco) and 1% of antibiotic-antimycotic solution (Sigma) at 37 °C and 5% CO₂. OECs were enriched by medium supplementation with Bovine Pituitary Extract (5µg/ml, Gibco) and Forskolin (2µg/ml, Sigma).

2.1.2. Human Umbilical Cord Perivascular Cells

HUCPVCs were isolated according to the procedure originally described by Sarugaser *et al.* [14]. Pieces of cord were dissected by first removing the epithelium of the umbilical cord section along its length to expose the underlying Wharton's jelly. Each vessel, with its surrounding Wharton's Jelly matrix, was then pulled away, after which the ends of each dissected vessel were tied together with a suture creating "loops" that were placed into a solution of 0.5–0.75 mg/mL collagenase (Sigma, St. Louis, MO) with phosphate-buffered saline (PBS, Invitrogen/Gibco, Carlsbad, CA). After 18 h, the loops were removed from the suspension, which was then diluted with PBS to reduce the viscosity of the suspension and centrifuged. Following the removal of the supernatant, cells were resuspended in culture media, α -MEM (Invitrogen/Gibco) supplemented with 10% FBS (Invitrogen/ Gibco) and 1% antibiotic/antimycotic (Sigma), counted using a hemocytometer and plated out in T75 flasks at a density of 4,000 cells/cm². The culture medium was changed every 2/3 days. Upon confluence, cells were trypsinized and passaged to new T75 flasks.

2.1.3. Bone Marrow-Derived Mesenchymal Stem Cells

BM-MSCs, acquired from Lonza (Switzerland), were cultured in α -MEM (Invitrogen/Gibco) supplemented with 10% FBS and 1% antibiotic-antimycotic mixture. The culture medium was changed every 2/3 days. Upon confluence, cells were trypsinized and passaged to new T75 flasks.

2.1.4. Human Adipose-Derived Adult Stem Cells

ASCs were isolated according to a protocol previously described by Dubois *et al.* [15]. All protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Research Boards (IRB) prior to the study. Liposuction aspirates from subcutaneous adipose tissue sites (abdomen, flank, thighs) were obtained from female subjects undergoing elective plastic surgical procedures. Tissues were then digested in a 0.1% collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) prewarmed to 37°C for 60 minutes after which they were centrifuged for 5 min at 300-500g at room temperature. The supernatant, containing mature adipocytes, was aspirated. The pellet was identified as the stromal vascular fraction (SVF). The SVF were suspended and plated immediately in T225 flasks in Stromal Medium (DMEM/F 12 Ham's, 10% fetal bovine serum (Hyclone, Logan, UT), 100 U penicillin/100 µg streptomycin/0.25 µg Fungizone) at a density of 0.156 ml of tissue digest/cm² of surface area for expansion and culture. After reaching confluence cells were passaged and kept in stromal medium.

2.2. Metabolic Activity Assay

Cell viability was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). This assay is based on the bioreduction of a tetrazolium compound (MTS), into a water-soluble brown formazan product. This conversion is accomplished by NADPH or NADH production by the dehydrogenase enzymes in metabolically active cells. Complete culture medium was replaced by standard DMEM/F12 medium containing MTS in a 5:1 ratio and incubated in a humidified

atmosphere at 37 °C and 5% CO₂. After 3h of incubation, the optic density for triplicates of each sample (n=3) was measured at 490 nm in a microplate reader.

2.3. Cell Proliferation Assessment

The cell proliferation rate was determined using the 5-bromo-2'-deoxyuridine assay (BrdU, Roche, USA), which quantifies the BrdU incorporation during DNA synthesis in replicating cells. To perform the assay, BrdU was added to the cell cultures for 24 hours and after that the medium was removed. The cells were then fixed and its DNA denatured with FixDenat (Roche, USA) in a single step, after which the anti-BrdU peroxidase antibody (Roche, USA) was added. The immune complexes were detected by the quantification of the substrate reaction product, measuring the optical density at 370 nm (reference filter set at 492 nm) in a multiplate reader (BioRad, USA).

2.4. Immunocytochemistry and phalloidin/DAPI staining

The following primary antibodies were used for the immunocytochemical studies: monoclonal mouse anti-GFAP (Chemicon) for astrocytes; monoclonal mouse anti-O4 (R&D Systems, Canada) for oligodendrocytes; and polyclonal rabbit anti-p75 (Millipore) for OECs. For all immunohistochemical procedures, the appropriate controls were obtained by omission of the relevant primary antibody. Cells on the substrates were fixed with PBS solution containing 4% PFA for 20 min (on glass) or 1 h (on the hydrogel) at room temperature and then washed with PBS. After cell membrane permeation (except for p75 and O4 antibodies) and blocking by treating with 0.3% Triton X-100 (Sigma, USA) and 10% of FBS solution at room temperature for 1 h, each specific primary antibody solution was added for 1h. After washing with 0.5% of FBS in PBS, the samples were exposed to the specific secondary antibody (Invitrogen) for 1h

and then washed with 0.5% FBS. Finally, cell nuclei were counterstained with 1 µg/ml DAPI (Invitrogen) for 1h.

For the phalloidin/DAPI staining, cells were fixed with 4% of paraformaldehyde for 30 min at room temperature and then treated with 0.3% TritonX-100. After PBS washing, 0.1µg/ml of phalloidin (Sigma) was added to the cells during 30 min. Finally, cell nuclei were counterstained with DAPI (1µg/ml, Invitrogen) for 10 min.

2.5. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences among groups were assessed by two-way ANOVA followed by a Bonferroni post-hoc test. A p-value of ≤ 0.05 (95% confidence level) was set as the criteria for statistical significance. All data are presented as mean \pm standard deviation.

3. Results

3.1. Metabolic Activity Assessment

The MTS test revealed that the OECs secretome affects the metabolic activity of mesenchymal stem cells in distinct time dependent manners. The mitochondrial activity of stem cells derived from the adipose tissue in co-culture with OECs was 1.4 fold higher ($p < 0.01$) than when ASCs were cultured alone (Figure 2a). However, this difference was only observed after 7 days of incubation. No differences were observed after two days of culture. On the contrary, for the stem cells derived from the bone marrow and from the umbilical cord it was possible to observe a significantly higher metabolic activity when co-cultured with OECs after 2 days of incubation. The

metabolic activity of co-cultured bmMSCs was 1.7-fold higher ($p<0.05$) than bmMSCs in monoculture (figure 2b), while the co-cultured HUCPVCs presented a 2.8 fold higher ($p<0.001$) activity than cells growing in monoculture (figure 2c) For these two cell populations no differences were observed between controls and co-cultures after 7 days. Moreover, it was not observed any detrimental effect of the OECs secretome on the metabolic activity of all adult stem cells used.

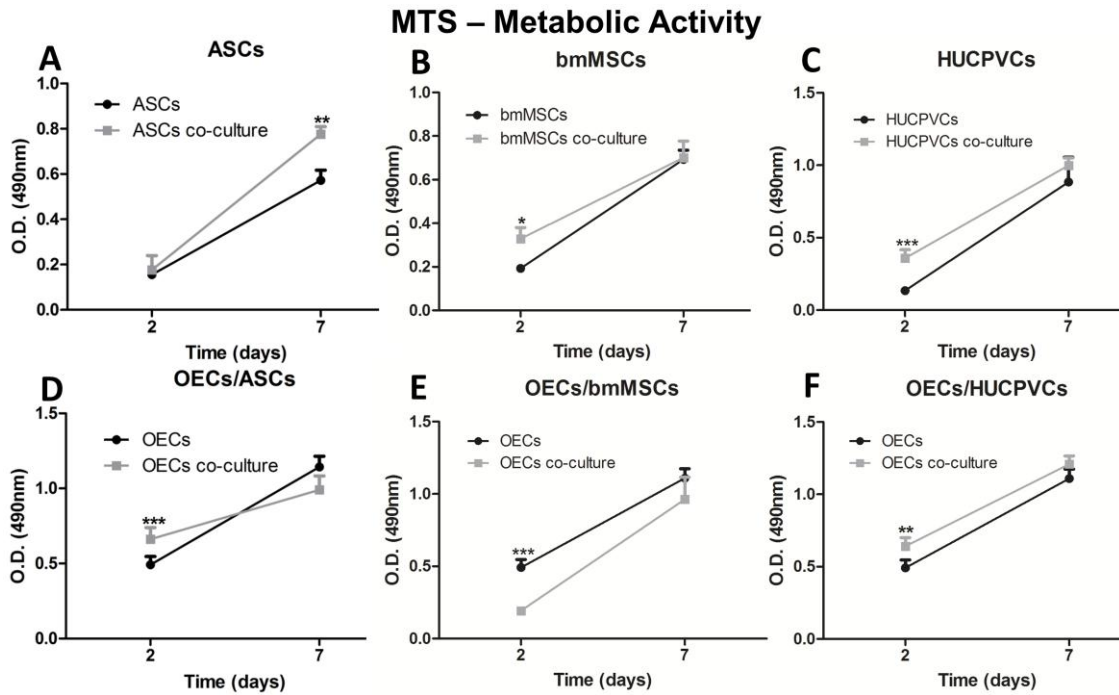


Figure VII.2. Metabolic activity of OECs and adult stem cells culture in transwell systems and in monoculture. Metabolic activity of (A) ASCs, (B) bmMSCs and (C) HUCPVCs in monoculture or in co-culture with OECs. Metabolic activity of OECs in monoculture or in co-culture with (D) ASCs, (E) bmMSCs and (F) HUCPVCs. Values are shown as mean \pm standard deviation ($n=3$ samples of 2×10^4 adult stem cells and 1×10^5 OECs/sample, * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

The study of the stem cells' secretome effects on the metabolic activity of OECs revealed that, after 2 days of culture, each stem cell's secretome produced different outcomes on the OECs activity. The metabolic activity of the OECs was 1.3-fold higher ($p<0.01$) when co-cultured with ASCs (Figure 2d) and HUCPVCs (Figure 2f) than

when in monoculture. In contrast, under the effects of the bmMSCs secretome the OECs presented a 2.5-fold lower ($p < 0.001$) metabolic activity than the control cells (Figure 2e). All the effects observed after 2 days of culture, either positive or negative, were not observed after 7 days of culture, as the metabolic activity of OECs was similar both on cells in co-culture and controls.

3.2. Proliferation Rate Evaluation

The BrdU incorporation during DNA synthesis in replicating cells was used to quantify the proliferation rate of the stem cells and OECs. Each of the stem cells was affected in a similar way by the OECs secretome. After 2 days of culture, all the distinct stem cells that were co-cultured together with the OECs presented significantly higher proliferation rates than the ones growing in monoculture. Co-cultured ASCs exhibited a 1.5-fold increase ($p < 0.01$) on the proliferation rate than the monoculture cells (figure 4a). Additionally, co-cultured bmMSCs and HUCPVCs presented an approximately 2.5-fold increase ($p < 0.001$) than monoculture cells (figure 4c and b). No differences in the proliferation rate of the distinct stem cells were detected after 7 days.

The study of the stem cells' secretome effect on the proliferation rate of OECs showed that just the secretome of HUCPVCs significantly influenced the OECs proliferation. Co-cultured OECs presented a 1.7-fold increase ($p < 0.01$) of BrdU incorporation than monocultured OECs (Figure 3f). This difference was observed only at the day 2 of co-culture since no differences were observed at day 7. OECs growing under the influence of the ASCs or bmMSCs secretome did not present significant differences when compared to OECs in monoculture both after 2 and 7 days (Figure 3d and 3e). Finally,

results show that both the stem cells and OECs secretomes do not negatively influence the proliferation rate of each other.

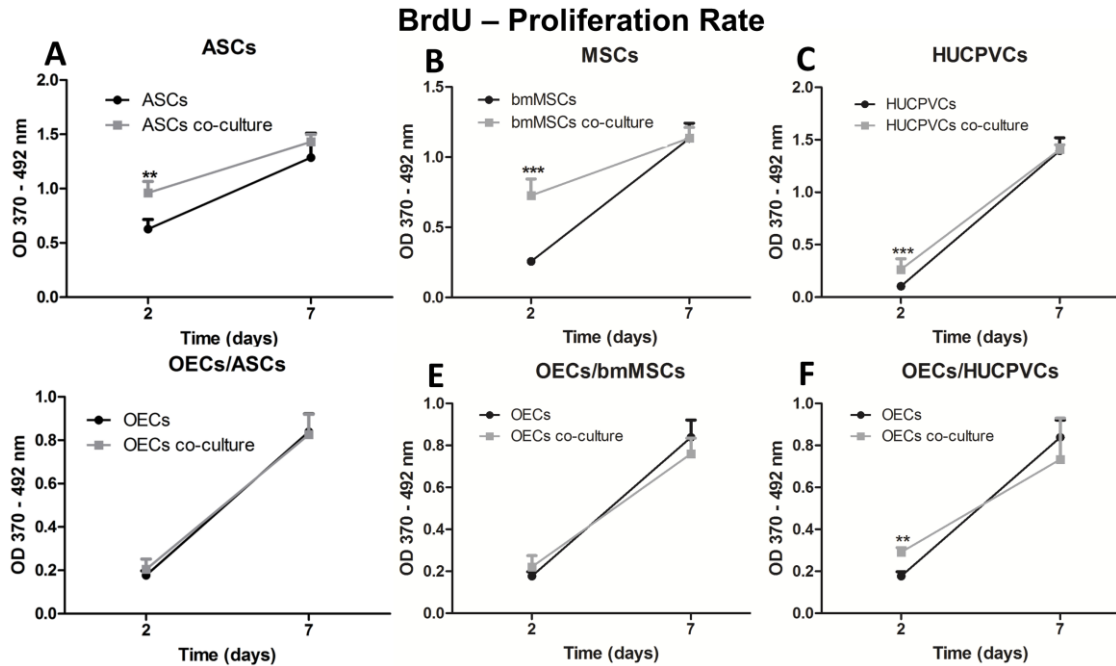


Figure VII.3. Proliferation rate of OECs and adult stem cells culture in transwell systems and in monoculture. Proliferation rate of (A) ASCs, (B) bmMSCs and (C) HUCPVCs in monoculture or in co-culture with OECs. Proliferation rate of OECs in monoculture or in co-culture with (D) ASCs, (E) bmMSCs and (F) HUCPVCs. Values are shown as mean \pm standard deviation ($n=3$ samples of 2×10^4 adult stem cells and 1×10^5 OECs/sample, ** $p<0.01$; *** $p<0.001$).

3.3. Stem Cells Differentiation, OECs Purity and Total Number of Cells

After 2 and 7 days in co-culture the stem cells and the OECs were subjected to an immunocytochemistry protocol in relation to the cell density and the stem cells differentiation. The proliferation studies revealed that the total number of ASCs and bmMSCs evolved from similar levels at day 2 to significantly higher cell densities at day 7, only when cells were under the influence of the OEC secretome. The number of co-cultured ASCs was 1.8-fold higher ($p<0.001$) than the monocultured ASCs (figure 4a). Moreover, bmMSCs conditioned by the OEC secretome were 1.2-fold more

($p < 0.05$) than bmMSCs in monoculture (figure 4b). In contrast, it was possible to observe that the factors produced by the OECs did not significantly affect the density of HUCPVCs (Figure 4c).

The factors produced by the stem cells revealed to have a positive effect on the OECs. The number of p75 positive cells (marker for OECs) was similar after 2 days of culture; however, after 7 days, the density of p75 positive OECs was significantly higher when these cells were co-cultured with ASCs or bmMSCs than in monoculture. The number of OECs conditioned by the ASCs' secretome was 2.0-fold higher ($p < 0.05$) than OECs growing solo (figure 4d). Additionally, OECs conditioned by bmMSCs' secretome was 1.6-fold higher than controls (figure 4e). This positive effect was not observed under the HUCPVCs secretome influence, in this circumstance, OECs did not significantly increase in number when compared to controls (Figure 4f).

It was possible to observe that the percentage of OECs in culture significantly decreased over time when in monoculture (Figure 4g-i, p75 positive cells of $50.7 \pm 5.1\%$ on the second day and $29.1 \pm 2.1\%$ on the seventh day of culture). In other words, this indicates that the culture became enriched for cells other than OECs. However, when OECs were co-cultured with each of the stem cell types, evidence of "contaminating" cell types was significantly reduced ($p < 0.05$). The percentage of OECs remained around 50% of the total number of cells in culture, while, the monocultured OECs decrease to around 29% (figure 4g-i). As stated above, the proliferation rate measured following BrdU incorporation did not show any significant difference between co-cultures and controls, in this sense, it is likely that the stem cells secretome selectively promoted the survival of the OECs, rather than the "contaminating" cells.

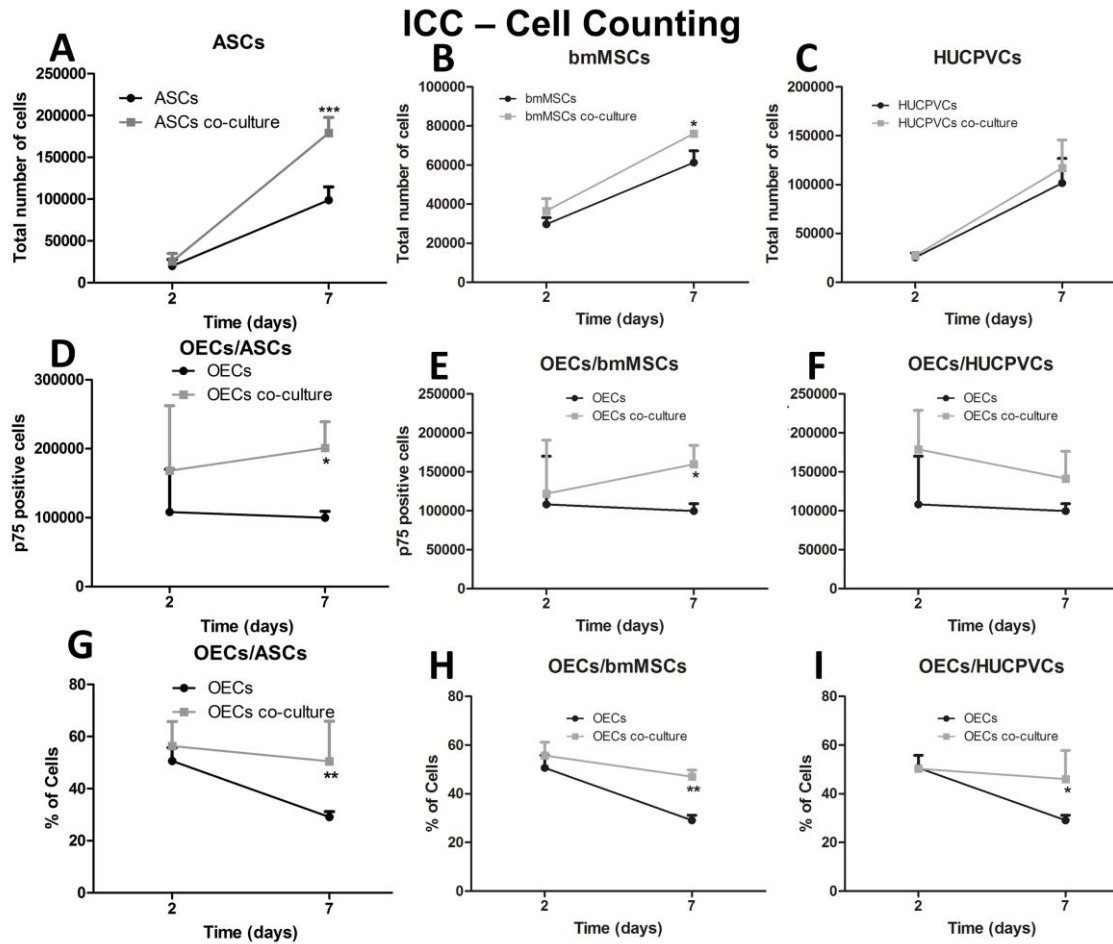


Figure VII.4. Immunocytochemistry counting of OECs and adult stem cells culture in transwell systems (gray line) and in monoculture (black line). Total number of (A) ASCs, (B) bmMSCs and (C) HUCPVCs in monoculture or in co-culture with OECs. Total number of p75-positive cells (OECs) in monoculture or in co-culture with (D) ASCs, (E) bmMSCs and (F) HUCPVCs. Percentage of OECs in monoculture or in co-culture with (D) ASCs, (E) bmMSCs and (F) HUCPVCs. Values are shown as mean \pm standard deviation ($n=3$ samples of 2×10^4 adult stem cells and 1×10^5 OECs/sample, * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Finally, immunocytochemistry studies revealed that the factors secreted by the OECs did not induce the differentiation of the adult stem cells herein assessed (Figure 5a, 5b and 5c). Moreover, the immunocytochemistry pictures also confirmed the results described above (Figure 6). The factors produced by the OECs significantly promoted proliferation of the ASCs (Figure 5d) and bmMSCs (Figure 5e) but not the HUCPCVs

(Figure 5f). Additionally, the factors secreted by each of the stem cells used promoted a higher purity of the OECs cultures (Figure 5g-j).

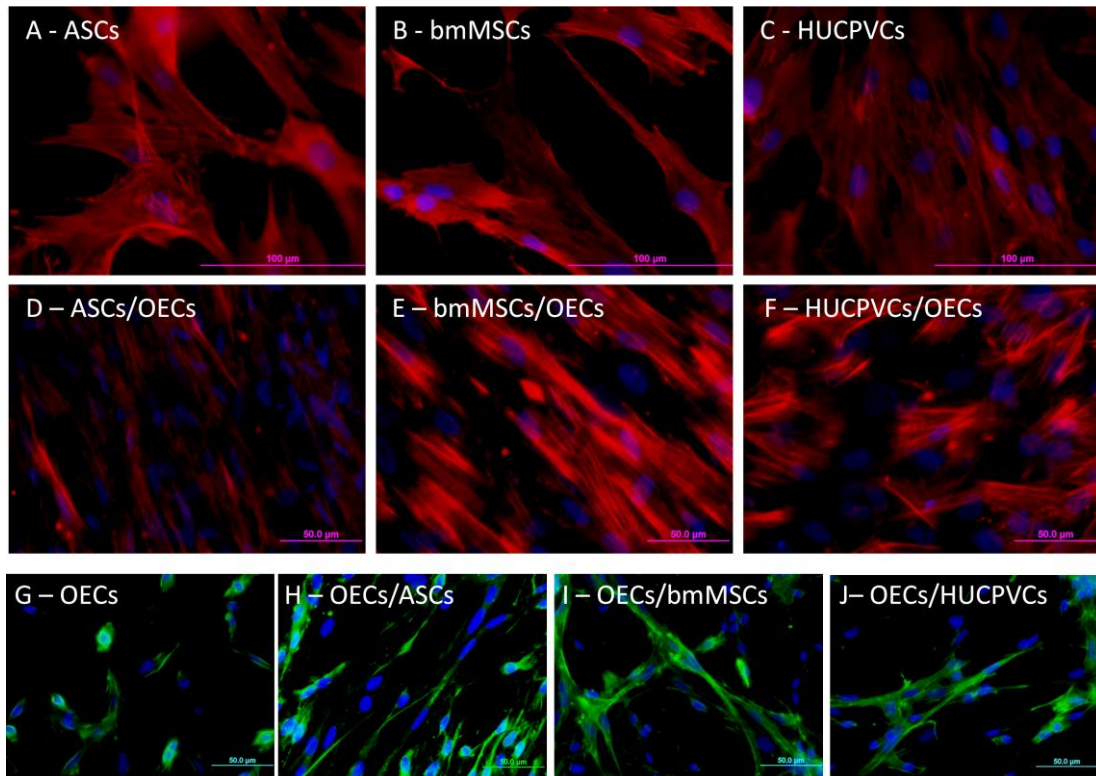


Figure VII.5. Images of fluorescence microscopy of adult stem cells and OECs either in monoculture or in transwell cultures. (**A-F**) The cytoplasm of adult stem cells was stained with the anti-F-actin dye phalloidin and nuclei counterstained with DAPI. (**G-J**) The OECs were identified with immunocytochemistry against p75 protein.

4. Discussion

The use of cells with the capacity to repair or substitute a damaged tissue holds great promise for the regenerative medicine field. Due to their *sui generis* characteristics, such as the ability to differentiate and replace damaged cells, to secrete trophic factors and to be easily obtained from human patients, adult stem cells are potential candidates for clinical treatments. In context of SCI, OECs hold particular promise for promoting regeneration. OECs are responsible for the continuous growth of new axons into the CNS tissue that takes place in the adult olfactory system [10]. Moreover, several authors

previously demonstrated that OECs have the ability to promote regeneration of CNS axons both *in vitro* and *in vivo* [16-19]. However most of the data obtained until now suggest that no single cell therapy will be sufficient to overcome all the biological complications caused by SCI. For this reason, in the work herein presented we study the possible positive or detrimental effects of the secretome of OECs and adult stem cells derived from the adipose, bone marrow and umbilical cord tissue.

The results herein presented show that the secretome of OECs exerts a positive effect on the metabolic activity and proliferation of mesenchymal stem cells from different origins (All results are summarized in table 1). However, it is possible to observe that this effect is higher for the ASCs than for the other stem cells, namely in proliferation. Moreover, the stem cells secretome also have beneficial effects in the metabolic activity and proliferation/survival of OECs. Again, the stem cells from adipose origin have a stronger impact on the OECs behavior than the other stem cells. In this sense, the most adequate cell type to be combined with the OECs in future studies would be the ASCs. It is important to point out that in future experiments, after the cell seeding (the stem cells on the SPCL layer, and the OECs on the hydrogel) and following the scaffold implantation in SCI animal models, the secreted factors produced by the stem cells will most likely influence the OECs behavior as well as the nervous cells from the injured tissue where the OECs are implanted. In this sense, the mesenchymal stem cells secretome can also be advantageous for the injured spinal cord site, promoting the growth and regeneration of damaged tissue. Previous studies demonstrated that the secretome of the stem cells used here have a beneficial effect on nervous cells. For instance, Ribeiro *et al.* previously reported that the secretome of bmMSCs increased the cell viability of hippocampal cultures as well as the number of neurons in culture [20]. Moreover, similar results were obtained using the conditioned medium of HUCPVCs

and ASCs in neuronal and glial cultures [21, 22]. To the best of our knowledge the results herein presented show for the first time the secretome interactions between OECs and ASCs, HUCPVCs and bmMSCs on the metabolic activity and proliferation of the cells.

Table 1: Summary of the secretome effects between OECs and adult stem cells.

	ASCs	OECs	MSCs	OECs	PVCs	OECs
Metabolic Activity	↑	↑	↑↑	↓↓	↑↑	↑
Proliferation Rate	↑↑	--	↑↑	--	↑↑	↑↑
Cell Number	↑	↑↑	↑	↑	--	--
Culture Purity	n/a	↑↑	n/a	↑↑	n/a	↑↑

Key: One arrow symbolizes a difference of 1.01 to 1.49 fold and two arrows symbolized a difference of 1.5 fold or higher on the parameter tested. The direction of the arrow (up or down) means positive or negative effects, respectively. Lines means no significantly different.

The explanation behind the positive effects of the OECs secretome on the stem cells behavior may be related with the ability of OECs to produce several neurotrophic factors. Woodhall *et al.* previously reported that OECs are capable to produce nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) [12]. The higher metabolic activity and proliferation rate presented by the stem cells may be consequence of the action of these factors. Moreover, these results are also in accordance with a previously work where we demonstrated that the OEC secretome is able to promote the proliferation of neural stem cells either in 2D and 3D culture [23].

The stem cells herein used are also able to secrete several factors. For instance, Rehman *et al.* previously reported that ASCs produce basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-

β) [24]. Additionally, previous studies also demonstrated that bmMSCs and HUCPVCs are capable to secrete factors such as BDNF, GDNF, VEGF and granulocyte-colony stimulating factor (G-CSF) [25, 26]. These factors secreted by the stem cells may explain why the OECs in co-culture presented a higher number of p75 positive cells. Furthermore, it was previously demonstrated that the presence of growth factors such as bFGF allow for primary cell cultures with higher percentage of OECs in relation to fibroblast [27]. This phenomena may explain our results, since the purity of OECs increased when cells were under the influence of the factors secreted by the stem cells. Nevertheless, even with a higher percentage of OECs in the co-culture group than in the controls, we only obtained ~50% of OECs in culture. This percentage is similar to what can be found in literature when OECs were used. For instance, Keyvan-Fouladi *et al.* demonstrated that a yielded cell cultures having around 50% OECs and 50% fibronectin⁺ olfactory nerve fibroblasts are able to promote functional repair of the corticospinal tract [28]. Moreover, Jani *et al.* proposed that the olfactory nerve fibroblasts may not be contaminants that needs to be removed but rather essential components of the cell suspension, that play a crucial role in the OECs growth and promoting its ability to graft [29].

It was only possible to find a single report using a similar strategy to the one that we used in this work. Wang *et al.*, co-culture OECs and ASCs and found out that in this conditions ASCs were able to differentiate in OECs-like cells [30]. These results contrast with the ones presented here, where no differentiation was detected for all the stem cells used, including the ASCs. The possible explanations for this difference may reside on the substrate where the ASCs were growing. In Wang's work, the authors seeded the ASCs on a collagen scaffold since they believe that the 3D culture is an

important factor in the ASCs differentiation. We believe that not only the 3D culture, but also the collagen scaffold may have influence on the ASCs differentiation.

Amemori *et al.* previously demonstrated that the co-transplantation of OECs and bmMSCs does not have synergistic effects after SCI in the rat compression model [31]. Interestingly, the only negative effect here observed was between the OECs and bmMSCs, namely the secretome of the bone marrow derived stem cells negatively affected the metabolic activity of the OECs. This may be one of the reasons for the absence of synergistic effects.

In Summary, the results herein described add new and important knowledge in the cell therapy field about the secretome effects between OECs and mesenchymal stem cells from adipose, bone marrow and umbilical cord origins. Further work will focus on the seeding of ASCs and OECs in the hybrid scaffold followed by implantation in SCI animal models.

5. Acknowledgements

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References

- [1] Choo AM, Liu J, Dvorak M, Tetzlaff W, Oxland TR. Secondary pathology following contusion, dislocation, and distraction spinal cord injuries. *Experimental Neurology*. 2008;212:490-506.
- [2] Tate CC, Shear DA, Tate MC, Archer DR, Stein DG, LaPlaca MC. Laminin and fibronectin scaffolds enhance neural stem cell transplantation into the injured brain. *Journal of Tissue Engineering and Regenerative Medicine*. 2009;3:208-17.
- [3] Johnson PJ, Tatara A, McCreedy DA, Shiu A, Sakiyama-Elbert SE. Tissue-engineered fibrin scaffolds containing neural progenitors enhance functional recovery in a subacute model of SCI. *Soft Matter*. 2010;6.
- [4] Kim H, Zahir T, Tator CH, Shoichet MS. Effects of Dibutyl Cyclic-AMP on Survival and Neuronal Differentiation of Neural Stem/Progenitor Cells Transplanted into Spinal Cord Injured Rats. *PLoS ONE*. 2011;6:e21744.
- [5] Silva NA, Salgado AJ, Sousa RA, Oliveira JT, Pedro AJ, Leite-Almeida H, et al. Development and Characterization of a Novel Hybrid Tissue Engineering Based Scaffold for Spinal Cord Injury Repair. *Tissue Engineering Part A*. 2010;16:45-54.
- [6] Silva NA, Sousa RA, Oliveira JT, Fraga JS, Fontes M, Cerqueira R, et al. Benefits of Spine Stabilization with Biodegradable Scaffolds in Spinal Cord Injured Rats. *Tissue Engineering Part C*. 2012;Epub ahead of print.
- [7] Rada T, Santos TC, Marques AP, Correlo VM, Frias AM, Castro AG, et al. Osteogenic differentiation of two distinct subpopulations of human adipose-derived stem cells: an in vitro and in vivo study. *Journal of Tissue Engineering and Regenerative Medicine*. 2012;6:1-11.
- [8] Martins A, Duarte ARC, Faria S, Marques AP, Reis RL, Neves NM. Osteogenic induction of hBMSCs by electrospun scaffolds with dexamethasone release functionality. *Biomaterials*. 2010;31:5875-85.
- [9] Baksh D, Yao R, Tuan RS. Comparison of Proliferative and Multilineage Differentiation Potential of Human Mesenchymal Stem Cells Derived from Umbilical Cord and Bone Marrow. *STEM CELLS*. 2007;25:1384-92.
- [10] Doucette R. Glial influences on axonal growth in the primary olfactory system. *Glia*. 1990;3:433-49.
- [11] Ramón-Cueto A, Avila J. Olfactory ensheathing glia: properties and function. *Brain Research Bulletin*. 1998;46:175-87.

- [12] Woodhall E, West AK, Chuah MI. Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors. *Molecular Brain Research*. 2001;88:203-13.
- [13] Silva NA, Sousa RA, Pires AO, Sousa N, Salgado AJ, Reis RL. Interactions between Schwann and olfactory ensheathing cells with a starch/polycaprolactone scaffold aimed at spinal cord injury repair. *Journal of Biomedical Materials Research Part A*. 2012;100A:470-6.
- [14] Sarugaser R, Ennis J, Stanford WL, Davies JE. Isolation, Propagation, and Characterization of Human Umbilical Cord Perivascular Cells (HUCPVCs). In: Audet J, Stanford WL, editors. *Methods in Molecular Biology*: Humana Press Inc; 2009. p. 269-79.
- [15] Dubois SG, Floyd EZ, Zvonic S, Kilroy G, Wu X, Carling S, et al. Isolation of human adipose-derived stem cells from biopsies and liposuction specimens. In: Prockop DJ, Phinney DG, Bunnell BA, editors. *Methods in Molecular Biology*: Springer; 2008. p. 69-79.
- [16] Jiao Y, Novozhilova E, Karlén A, Muhr J, Olivius P. Olfactory ensheathing cells promote neurite outgrowth from co-cultured brain stem slice. *Experimental Neurology*. 2011;229:65-71.
- [17] Chuah MI, Hale DM, West AK. Interaction of olfactory ensheathing cells with other cell types in vitro and after transplantation: Glial scars and inflammation. *Experimental Neurology*. 2011;229:46-53.
- [18] Li Y, Field PM, Raisman G. Repair of Adult Rat Corticospinal Tract by Transplants of Olfactory Ensheathing Cells. *Science*. 1997;277:2000-2.
- [19] Ramón-Cueto A, Cordero MI, Santos-Benito FF, Avila J. Functional Recovery of Paraplegic Rats and Motor Axon Regeneration in Their Spinal Cords by Olfactory Ensheathing Glia. *Neuron*. 2000;25:425-35.
- [20] Ribeiro CA, Salgado AJ, Fraga JS, Silva NA, Reis RL, Sousa N. The secretome of bone marrow mesenchymal stem cells-conditioned media varies with time and drives a distinct effect on mature neurons and glial cells (primary cultures). *Journal of Tissue Engineering and Regenerative Medicine*. 2011;5:668-72.
- [21] Salgado AJ, Fraga JS, Neves NM, Reis RL, Sousa N. Role of paracrine factors released by mesenchymal progenitors from the umbilical cord in neurons/glia cell viability, proliferation and differentiation TERMIS EU, Porto: *Tissue Engineering Part A*. May 2008, 14(5): 691-943. p. 695.

- [22] Ribeiro CA, Salgado AJ, Fraga JS, Neves NM, Gimble JM, Reis RL, et al. A comparative study on the effects of adipose tissue derived and bone marrow mesenchymal stem cells on neurons/glia cells viability, proliferation and differentiation. *TERMIS EU, Porto: Tissue Engineering Part A*. May 2008, 14(5): 691-943.
- [23] Silva N, Cooke M, Tam R, Sousa N, Salgado AJ, Reis RL, et al. The Effects of Peptide Modified Gellan Gum and Olfactory Ensheathing Glia Cells on Neural Stem/Progenitor Cell Fate. *Biomaterials*. 2012;accepted for publication.
- [24] Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, et al. Secretion of Angiogenic and Antiapoptotic Factors by Human Adipose Stromal Cells. *Circulation*. 2004;109:1292-8.
- [25] Koh S-H, Kim KS, Choi MR, Jung KH, Park KS, Chai YG, et al. Implantation of human umbilical cord-derived mesenchymal stem cells as a neuroprotective therapy for ischemic stroke in rats. *Brain Research*. 2008;1229:233-48.
- [26] M. Carvalho M, G. Teixeira F, L. Reis R, Sousa N, J. Salgado A. Mesenchymal Stem Cells in the Umbilical Cord: Phenotypic Characterization, Secretome and Applications in Central Nervous System Regenerative Medicine. *Current Stem Cell Research & Therapy*. 2011;6:221-8.
- [27] Kawaja MD, Boyd JG, Smithson LJ, Jahed A, Doucette R. Technical Strategies to Isolate Olfactory Ensheathing Cells for Intraspinal Implantation. *Journal of Neurotrauma*. 2009;26:155-77.
- [28] Keyvan-Fouladi N, Raisman G, Li Y. Functional Repair of the Corticospinal Tract by Delayed Transplantation of Olfactory Ensheathing Cells in Adult Rats. *The Journal of Neuroscience*. 2003;23:9428-34.
- [29] Jani HR, Raisman G. Ensheathing cell cultures from the olfactory bulb and mucosa. *Glia*. 2004;47:130-7.
- [30] Wang B, Han J, Gao Y, Xiao Z, Chen B, Wang X, et al. The differentiation of rat adipose-derived stem cells into OEC-like cells on collagen scaffolds by co-culturing with OECs. *Neuroscience Letters*. 2007;421:191-6.
- [31] Amemori T, Jendelová P, Růžicková K, Arboleda D, Syková E. Co-transplantation of olfactory ensheathing glia and mesenchymal stromal cells does not have synergistic effects after spinal cord injury in the rat. *Cytotherapy*. 2010;12:212-25.

SECTION IV

CHAPTER VIII

General Conclusions and Final Remarks

Chapter VIII.

General Conclusions and Final Remarks

Repair of the injured spinal cord is probably the “holy grail” of regenerative medicine. The development of strategies to protect and repair the injured spinal cord has been facilitated by the identification of key mechanisms of secondary injury. Due to the nature of the injury, it is of general consensus that successful functional recovery will not simply rely on a single therapeutic approach. Future clinical regenerative approaches will most likely include a combination of multiple strategies. In this sense, the work developed in the scope of this thesis was based in the concepts of tissue engineering. This new field of science merges the knowledge from physics, materials science, engineering, chemistry, biology, and medicine in an integrated manner with the ultimate goal to restore, maintain, or improve tissue function. Based on these concepts, the generation of a novel potential tool to help in the solution of spinal cord injuries was pursued.

Firstly, a novel hybrid biodegradable 3D scaffold was developed and characterized. This biphasic scaffold was comprised of two natural polysaccharide-based biomaterials: starch/poly- ϵ -caprolactone (SPCL) blend and gellan gum. The choice for natural-based materials relies in the advantages they may ultimately confer to the performance of the tissue engineered construct. Therefore, natural materials frequently exhibit better

integration, less cytotoxicity, and most are present or display structural affinity with the native tissue.

SPCL has been originally proposed by Reis and colleagues for a wide range of applications, retrieving promising results, particularly, in bone tissue engineering. In our concept, the SPCL tubular structure assures mechanical stability to the entire construct, namely by establishing a connection to the adjacent vertebral bone. In this sense, the SPCL phase is aimed at mimicking the bone functions. Alternatively, gellan gum hydrogel is aimed as a cell encapsulation system to support axonal regeneration in the injured spinal cord. Gellan gum was originally proposed by our group for cartilage tissue engineering. It is a FDA approved material that can be prepared and mixed with cells/drugs using very simple techniques and using as reagents only water and PBS. Gellan gum can be injected in a minimally-invasive way to form a gel *in situ*, thereby not being harmful to cells and the surrounding tissues. Moreover, it possesses a carboxylic group in the glucuronic residue that may be modified to confer improved functions.

The SPCL 3D structure was developed by rapid prototyping. This processing technique was quite versatile allowing for the development of tubular scaffolds with configurable outer layer thickness, fiber/filament orientation and pore geometry. The SPCL scaffolds also possessed configurable mechanical properties and high interconnectivity. Then, we demonstrated that SPCL/gellan gum hybrid structures were non-cytotoxic and disclosed an apparent biocompatible response *in vivo*. It was also possible to observe that these structures allowed the *in vitro* culturing of oligodendrocytes for periods up to one week.

After the development, the physical and biological characterization, the biocompatible tests of the hybrid structures, and the *in vivo* proof of concept of the methodology herein proposed was performed. The hemisection model was used due to its feasibility for strategies that aim at axonal regeneration. Moreover, since the integrity and function of one side of the cord is preserved, the bladder and bowel function are maintained, resulting in less *post-operative* care and less animal death. The results showed that SPCL scaffolds are suitable structures to promote spine stabilization. Moreover, SPCL also provided support for bone ingrowth. However, the most important achievement of this work was the disclosure, for the first time, of a correlation between spine stabilization and motor recovery in SCI rats. The motor evaluation was assessed by three different tests, reinforcing in this way the conclusions obtained. These results suggests that researchers currently testing treatments for SCI repair might have to take into account the use of spine stabilization in combination with their approaches.

In the *in vivo* proof of concept experiment, it was also employed the gellan gum hydrogel. However, this material failed to demonstrate any beneficial outcome in the motor behavior of SCI rats (data not shown). In order to answer this drawback, two different experimental methodologies were designed. In the first one, we studied the ability of SPCL scaffolds to support the 3D culture of OECs and SCs. It was previously demonstrated that OECs and SCs are able to secrete neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor and glia cell line-derived neurotrophic factor. Moreover, it is known that these factors have a positive effect on axonal growth. In this sense, combining the secretome effects of OECs and SCs with the previously observed beneficial effects of spine stabilization provided by the SPCL scaffolds implantation may lead to further locomotor recovery. The results demonstrated that

starch-based scaffolds were able to support the growth and migration of OECs and SCs. Additionally we demonstrated the importance of studying the long-term *in vitro* incubation periods on the number of cells that could be transplanted within the scaffold.

The second experimental work designed to improve the poor performance of gellan gum in the *in vivo* test was the chemical modification of the gel. In order to better mimic the properties of the ECM, gellan gum was chemically conjugated with the fibronectin-derived peptide sequence GRGDS via Diels-Alder click chemistry. Then, the impact of GRGDS-modified gellan gum (GRGDS-GG) on cell fate was studied. Amino acid analysis demonstrated the gellan gum was successfully conjugated with GRGDS. Furthermore, the GRGDS-gellan gum had a profound effect on neural stem/progenitor cell (NSPC) morphology and proliferation, distinct from the one observed in regular gellan gum, demonstrating the importance of GRGDS for cell-gellan gum interaction. The simplicity and broad applicability of the Diels-Alder click chemistry can be easily extended to other biomolecules to further promote cell differentiation and proliferation, for a better integration in the host tissue. In this work, we also demonstrated NSCs interacted extensively with OECs and showed significant survival and proliferation in relation to monocultures of NSCs alone. This combination strategy could be powerful, with OECs and gellan gum providing a cellular pathway on which NSCs could differentiate, thereby replacing the damaged tissue and achieving greater functional repair.

In the last work developed on the scope of this thesis, we studied the effects of OECs secretome on the metabolic activity and proliferation of ASCs, bmMSCs and

HUCPVCs. The stem cells' secretome effects on metabolic activity and proliferation of the OECs were also considered. The rationale beyond this study lies in the fact that mesenchymal stromal cells and OECs will be used in our hybrid concept. Adult stem cells will be seeded in the SPCL scaffolds, in order to improve bone repair, and OECs will be encapsulated in the gellan gum, in order to foster axonal regeneration. In this sense, our therapeutic approach puts in close contact both the mesenchymal stem cells and the OECs, allowing the secreted factors by these cells to diffuse and interact with each other. Results revealed that the secretome of OECs have a positive effect on the metabolic activity and proliferation of mesenchymal stem cells from different origins, being the ASCs the most positively affected. Moreover, the stem cells' secretome also have a beneficial effect on the metabolic activity and proliferation/survival of OECs. Moreover, the results obtained in this work add new and important knowledge in the cell therapy field about the secretome effects between OECs and mesenchymal stem cells from adipose, bone marrow and umbilical cord origins.

As final remarks, it can be stated that the work performed and included in this thesis provided interesting studies on the potential use of our concept in the SCI field. More research studies should be performed by other research units to strengthen and validate this data. Importantly, the knowledge about the consequences of spine stabilization of SCI animal models was originally demonstrated in this thesis and may have an enormous impact in the field. Moreover, the synthesis of a gellan gum chemically conjugated with furfurylamine was also first reported in this thesis. This modification allows now researchers to conjugate the gellan gum with a wide range of factors/drugs via Diels-Alder click chemistry. Finally, important understanding was herein created

about biological/molecular interactions between OECs and adult stem cells obtained from adipose, bone marrow, umbilical cord, and nervous tissue.

